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THE HEDGEHOG PATHWAY IN RETINAL DEVELOPMENT AND DISEASE

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THE HEDGEHOG PATHWAY IN RETINAL DEVELOPMENT AND DISEASE

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Dedication

This work is dedicated to my family, who has supported me and has been my motivation throughout.

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I would like to thank the members of the Gross Lab, the Molecular Cell and Developmental Biology department, and the Institute of Cell and Molecular Biology at the University of Texas at Austin. I especially would like to thank my advisor, Jeff Gross, PhD., for his support over the years and for providing me with the opportunity and the freedom to pursue my research interests, and to the members of my committee for their advice, guidance and support.

THE HEDGEHOG PATHWAY IN RETINAL DEVELOPMENT AND DISEASE

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The Hedgehog receptor Patched (Ptch) is a well-studied tumor suppressor. Mutations in *Ptch* have been linked to mis-regulation of stem cell proliferation and tumorigenesis in numerous contexts. To study the role of Ptch function during retinal development and homeostasis, I analyzed retinal growth and patterning in the embryonic and post-embryonic (juvenile) zebrafish *ptc2* mutant line. *ptc2* deficiency in zebrafish results in an expansion of the stem/progenitor population of the ciliary marginal zone (CMZ), as well as ectopic proliferation within the neural retina at juvenile stages. *ptc2*$^{-/-}$ mutants also possess vitreo-retinal abnormalities that appear to be embryonic in origin. These phenotypes are similar to the ocular abnormalities previously reported in human patients suffering from Basal Cell Naevus Syndrome (BCNS), a disorder that has been linked to mutations in the human *PTCH* gene (the orthologue of the zebrafish *ptc2*), and point to the utility of the *ptc2* mutant line as a model for the study of BCNS-related ocular pathologies. In addition, peripheral retinal dysplasias that include ectopic neuronal clusters and disrupted lamination were observed at later, juvenile stages. It has

been previously proposed that retinal over-proliferation might contribute to retinal dysplasias observed in the post-natal *Ptch1*$^{+/-}$ mice (an established model for BCNS); however, this potential relationship has yet to be established experimentally. I demonstrated that a population of ectopically proliferating cells give rise to the ectopic neuronal clusters in the INL of *ptc2*$^{-/-}$ mutants and established ectopic proliferation as the likely cellular underpinning of retinal dysplasia in juvenile *ptc2*$^{-/-}$ mutants.

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CHAPTER I

Introduction: Molecular and Cellular Regulation of Retinal Development and Homeostasis

I.1 DEVELOPMENT OF THE VERTEBRATE EYE

Visual impairments affect over 160 million people worldwide, of which 37 million are blind (Resnikoff, Pascolini et al. 2004). Model organisms with similar physiology to humans are vital to understand underlying developmental processes, identify potential causative genes for human disorders, and develop therapies. The zebrafish has long been recognized as a useful model for the study of human ocular development and disease (Bilotta and Saszik 2001; Goldsmith and Harris 2003; Fadool and Dowling 2008; Gross and Perkins 2008; Bibliowicz, Tittle et al. 2011). Detailed characterization of the embryonic development of the zebrafish retina has not only shed light on the sequence of events in vertebrate retina development, but has also highlighted the similarities in the architecture of the zebrafish retina to that of the human retina (Schmitt and Dowling 1999; Lister 2002).

In zebrafish, eye development is rapid. The optic vesicle, which will ultimately give rise to the neural retina and the retinal pigment epithelium (RPE), evaginates from the forebrain at around 12 hours post fertilization (hpf) and remains attached to and

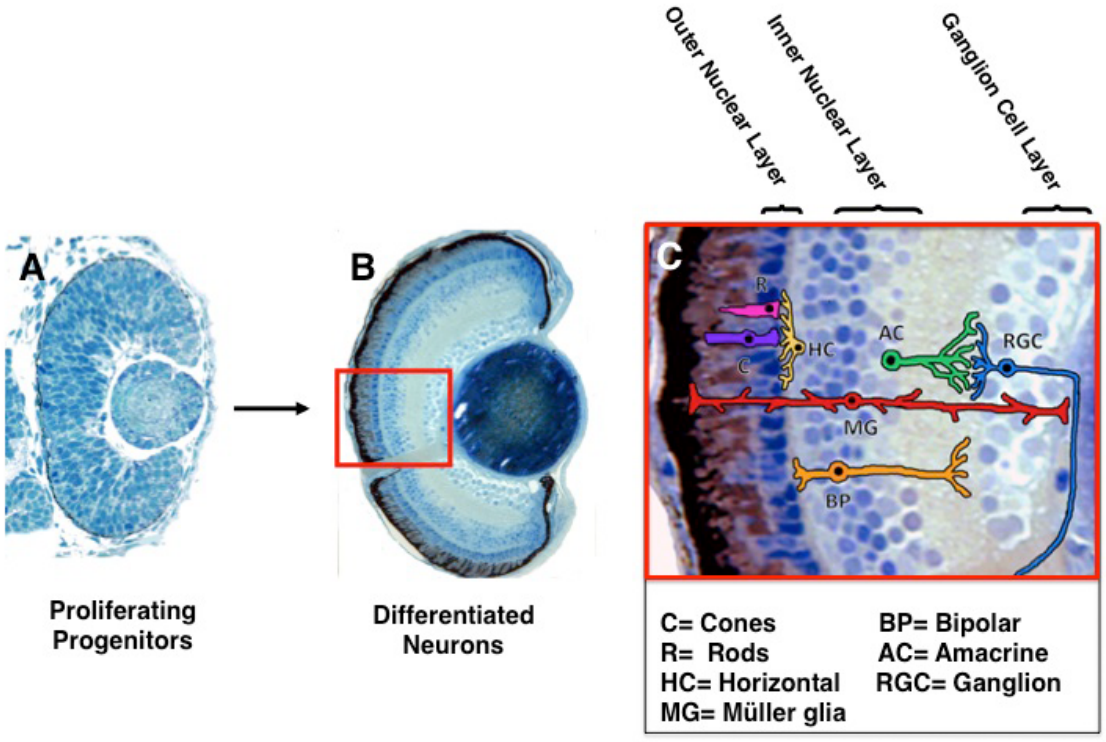

Figure I-1. The structure and cellular composition of the embryonic zebrafish retina. In zebrafish, a population of proliferative retinal progenitors (24hpf, A) give rise to a laminated and functional retina (5dpf, B). The differentiated retina is composed of three nuclear layers (C)- the ganglion cell layer (GCL), the inner nuclear layer (INL), and outer nuclear layer (ONL). The GCL is composed mainly of retinal ganglion cells, while the INL is made up of three types of neurons (horizontal cells, amacrine cells, and bipolar cells) and one type of glia (Müller glia). The ONL is composed of cone and rod photoreceptors.

continuous with the forebrain through a transient structure called the optic stalk. The optic vesicle then gives rise to the optic cup through a series of morphogenetic events that occur from about 16hpf to 20hpf (Schmitt and Dowling 1994). Neurogenesis begins at 28hpf and by as early as 72hpf zebrafish embryos exhibit visual function (Easter and Nicola 1996). In lower vertebrates, such as fish, growth of the mature retina continues at the retinal margin throughout the lifetime of the animal through the continual proliferation and subsequent differentiation of retinal progenitors at the ciliary marginal zone (CMZ, see section I.4).

As in humans, the mature zebrafish retina is composed of three nuclear layers separated by two plexiform layers (Figure I-1). Zebrafish possess four types of cones (blue, UV, and red/green double cones) and one rod cell type (Robinson, Schmitt et al. 1993). Rod and cone cell bodies reside in the outer nuclear layer (ONL), while the inner nuclear layer (INL) is occupied by amacrine, horizontal, bipolar cells, and Müller glia. Visual signals originating in the photoreceptors are transmitted through the retina to the ganglion cells, which make up the ganglion cell layer (GCL); their axons then relay the signal to the brain (Dowling 1987; Schmitt and Dowling 1999).

I.2 MOLECULAR REGULATION OF VERTEBRATE RETINAL DEVELOPMENT

I.2.1 Vertebrate retinal development

Retinal neurogenesis occurs via the coordination of proliferation, cell cycle exit, and differentiation and is regulated by both intrinsic and extrinsic factors (reviewed in (Agathocleous and Harris 2009)). Proliferation of the retinal neuroepithelium, which gives rise to the differentiated retina, is tightly controlled by components of the cell cycle machinery. Loss of CyclinD1, for example, results in reduced proliferation and decreased retinal size (Ma, Papermaster et al. 1998), while inactivation of the cell cycle inhibitors $p27^{kip1}$ and $p57^{kip2}$ results in ectopic proliferation (Dyer and Cepko 2000; Levine, Close et al. 2000). In addition, various transcription factors, including the homeodomain transcription factors Chx10, Six3, Pax6 and Prox1 have been shown to influence the proliferative properties of retinal progenitors (Marquardt, Ashery-Padan et al. 2001; Casarosa, Amato et al. 2003; Dyer, Livesey et al. 2003; Green, Stubbs et al. 2003). These transcription factors are thought to affect retinal progenitor proliferation by cross-regulating each other, and by regulating components of the cell cycle machinery (Zuber, Gestri et al. 2003). For example, $p27^{kip1}$ is activated upon loss of Chx10, resulting in cell cycle exit of progenitor cells, and leading to a hypocellular retina, suggesting a regulatory interaction between Chx10 and regulation of the G1 phase of the cell cycle (Green, Stubbs et al. 2003). Chx10 also regulates the transcriptional levels of a related homeobox gene, *Vsx1*, by directly binding to its promoter and ultimately affecting the proliferative properties of retinal progenitors (Clark, Yun et al. 2008).

Cell cycle progression in the developing retina also depends on the process of interkinetic nuclear migration (INM) (Baye and Link 2008). Nuclei of retinal progenitors within the proliferative neuroepithelium oscillate between the apical and basal surfaces of the neuroepithelium. Progenitors occupying the M-phase of the cell cycle are located at

the apical surface while those occupying the G1-, G2-, and S-phases are found more basally. Chemical inhibition of cell cycle progression results in an arrest in INM, and conversely, inhibition of INM migration results in inappropriate mitosis throughout the neuroepithelium (Ueno, Katayama et al. 2006). Supporting the link between cell polarity and migration with cell cycle progression are phenotypes of zebrafish mutants in which polarity and/or migration are defective. In the N-cadherin (*nok*) and *nagie oko* (*nok*) mutants, in which cell polarity is defective, the transition between proliferative progenitors and neurogenesis is defective, resulting in hyperplasia of retinal tissue (Yamaguchi, Imai et al. 2010). In the *mikre oko* (*mok*) mutant, apical migration is defective due a mutation in the dynactin-1 protein, resulting in premature cell cycle exit due to lack of Notch signaling originating at the apical surface of the neuroepithelium (Del Bene, Wehman et al. 2008). These results demonstrate a complex mechanism of cell cycle progression and cell cycle exit that is dependent on the orchestrated activity of the cell cycle machinery, cell migratory behavior, and extrinsic developmental cues.

Retinal cell fate decisions are also tightly linked to regulation of the cell cycle (Ohnuma, Hopper et al. 2002). Lineage analyses performed in several model species have established that the different cell types in the retina originate from a common pool of multipotent retinal progenitors (Turner, Snyder et al. 1990). How cell fate decisions within the retina are dictated is currently not fully understood, however a 'competence' model has emerged, in which retinal progenitors pass through a series of 'competence states' during which each retinal progenitor is competent to differentiate into a subset of cell types (Cepko, Austin et al. 1996). Like proliferation, cell fate decisions are dictated by both intrinsic and extrinsic factors, with competence defined intrinsically and the final

differentiation and cell fate decision promoted by extrinsic factors (reviewed in (Livesey and Cepko 2001; Stenkamp 2007)).

The contribution of intrinsic factors to cell fate decisions has been supported by gene profiling of single retinal progenitors that highlighted the heterogeneity of gene expression within a pool of retinal progenitors (Trimarchi, Stadler et al. 2008), and analyses of gene function in the retina have identified transcription factors that are required for the formation of specific retinal cell types (Ohsawa and Kageyama 2008). In addition, genetic and biochemical manipulations of developmental pathways such as Notch (Scheer, Groth et al. 2001; Del Bene, Wehman et al. 2008), Wnt (Kubo, Takeichi et al. 2005) and FGF (McFarlane, Zuber et al. 1998; Patel and McFarlane 2000), have supported the contribution of extrinsic signals in retinal cell fate decisions. While various factors that control neurogenesis have been identified, the relationship between intrinsic factors and the extrinsic developmental pathways that have so far been identified to control retinal neurogenesis are still incompletely understood.

I.2.2 Developmental pathway regulating retinal neurogenesis

Several developmental pathways have been shown to affect both proliferation and differentiation in the vertebrate retina. The role of Wnt signaling in controlling retinal proliferation has been demonstrated in several studies (Kubo, Takeichi et al. 2005; Denayer, Locker et al. 2008). It has been suggested that Wnt signaling might control proliferation through the direct regulation of cyclin D1 (Shtutman, Zhurinsky et al. 1999; Masai, Yamaguchi et al. 2005). However, Wnt signaling has also been shown to promote differentiation by indirectly regulating proneural genes through the control of both the

homeobox transcription factor Sox2 and activity of the Notch pathway (Kubo, Takeichi et al. 2005). Modulations of Wnt activity levels have therefore been implicated in the transition between retinal proliferation and differentiation (Agathocleous, Iordanova et al. 2009). Notch activity, in turn, has also been implicated in the inhibition of neuronal differentiation in the vertebrate retina, as over-expression of the Notch ligand, Delta, in the Xenopus retina resulted in a bias towards earlier cell fates (RGCs) (Dorsky, Chang et al. 1997). Misexpression of activated Notch in retinal progenitors result has an opposite effect, resulting in prolonged proliferation and lack of differentiation (Austin, Feldman et al. 1995; Bao and Cepko 1997). In zebrafish, inhibition of the Notch activity using γ-secretase inhibitors or through the *mib* mutation, which disrupt ubiquitin E3 ligase activity, result in loss of Müller glia, and support the role of Notch in promoting gliogenesis (Bernardos, Lentz et al. 2005). The glial cell fate promoting function of Notch has been shown to involve the activity of the cell cycle inhibitor $p27^{xic1}$(Ohnuma, Philpott et al. 1999). Taken together, these studies, along with others, support a role for the Notch pathway in promoting progenitor identity and glial cell fates in the retina (Furukawa, Mukherjee et al. 2000; Scheer, Groth et al. 2001).

Interestingly, *mib* mutants also present with disrupted retinal lamination that appears to be confined to the INL (Bernardos, Lentz et al. 2005). The correlation of disrupted lamination in *mib* mutants with the absence of differentiated Müller glia suggests that lack of proper Müller glia development underlies the lamination defects in these mutants. Indeed, *in vitro* studies utilizing cultured chick retinal cells have demonstrated that Müller glia provide the structural scaffolding required for proper retinal lamination (see section I.3) (Willbold, Rothermel et al. 2000). This possibility is further supported by earlier studies showing that abnormal Müller glia morphology that

results from exposure to exogenous Shh in retinal explants contributes to disorganization of retinal lamination (Wang, Dakubo et al. 2002).

FGF signaling has been shown to initiate retinal differentiation in the zebrafish retina. Analysis of retinal differentiation in *fgf8*/*fgf3* double mutants or after inhibition of FGF signaling with the chemical inhibitor SU5402 just prior to the onset of the first wave of RGC differentiation prevents the expression of *ath5*, a proneural gene required for RGC differentiation (Martinez-Morales, Del Bene et al. 2005). Studies in *Xenopus* demonstrated the additional role of FGF signaling in cell fate decisions during retinal development. Expression of a dominant-negative form of the *Xenopus* FGF receptor (FGFR) resulted in a 50% reduction in both amacrine cells and photoreceptors and a 3.5-fold increase in Müller glia, the last-born retinal cell type (McFarlane, Zuber et al. 1998). Over-expression of FGF2 ligand resulted in a 35% increase in RGCs and 50% increase in Müller glia (Patel and McFarlane 2000). The ratio of cone to rod photoreceptors was also influenced, and in a separate study the over-expression of a dominant-negative FGFR4a in retinal progenitors resulted in complete loss of photoreceptors (Zhang, El-Hodiri et al. 2003).

While genetic and molecular perturbations of specific signaling pathway have shed light on their specific roles during retinal neurogenesis, the interactions between these pathways are still poorly understood. The studies mentioned thus far reveal the complex nature of regulatory mechanisms during retinal development and highlight the need for the continued investigation of the molecular processes controlling cell behaviors in the developing retina.

I.2.3 The Hh pathway in embryonic retinal development

Hh proteins are secreted signaling molecules that act to control various developmental processes, including pattern formation, cell fate specification, and cell proliferation (Ingham and McMahon 2001). In vertebrates, binding of Hh to its 12-transmembrane receptor Patched (Ptch) ultimately results in the activation of Gli transcription factors and consequently in the expression of Hh target genes (Marigo, Davey et al. 1996; Stone, Hynes et al. 1996). In the absence of Hh ligands, Ptch inhibits the 7-transmembrane domain protein Smoothened (Smo), which in turn is responsible for transducing the Hh signaling cascade (Murone, Rosenthal et al. 1999). Upon binding of Hh to Ptch, inhibition on Smo is relieved, and the Hh transcriptional response is activated (Ingham 1998).

How Hh signaling results in diverse cellular responses required for its various developmental roles is still incompletely understood; however, part of the answer is thought to lie in the regulation on the levels of the Hh receptor Ptch. Hh signaling results in the transcriptional induction of Ptch, which in turn acts as a negative regulator of the pathway. Therefore, constant Hh signaling ultimately results in a 'de-sensitization' of the signal receiving cell to the Hh signal (Ingham and McMahon 2001). The cell's 'perception' of the amount of Hh signaling it receives has been proposed to rely on the absolute amount of unliganded, and therefore active, Ptch molecules (Taipale, Cooper et al. 2002). However, a second study proposed a model in which the ratio of unliganded (active) to liganded (inactive) Ptch determines cellular response to Hh (Casali and Struhl 2004). Also, in addition to the amount of Hh signaling, a cell is able to adapt its Hh-dependent transcriptional activity in response to the duration of Hh signaling it receives

(Dessaud, Yang et al. 2007)Together with desensitization of the cell to Hh signaling due to increased Ptch expression in response to Hh signaling (Hidalgo and Ingham 1990), these mechanisms are thought to account for a cell's ability to react to a wide range of Hh morphogen levels.

How Ptch functions to inhibit the activity of Smo also remains unclear. Ptch has been proposed to function as a transporter of small molecules across the cell membrane (Taipale, Cooper et al. 2002). In support of this model, Smo activity can be either activated or inhibited using various small molecules (Frank-Kamenetsky, Zhang et al. 2002). The structural similarity between one such compound, cyclopamine, to sterol point to sterols as potential regulators of Smo activity (Cooper, Porter et al. 1998). Interestingly, disruptions of cholesterol synthesis results in embryonic defects that phenocopy defective Hh signaling (Chiang, Litingtung et al. 1996). Additionally, the Ptch protein shares a high level of homology with NPC1, a cholesterol trafficking protein (Davies, Chen et al. 2000). In a more recent study, vitamin D3 levels were elevated in Ptch-transfected cells, and were shown to bind Smo *in vitro* (Bijlsma, Spek et al. 2006). These data, together with the reported ability of vitamin D3 to compete with cyclopamine for Smo binding (Chen, Taipale et al. 2002), supports vitamin D3 as a candidate endogenous molecule for Ptch-dependent inhibition of Smo.

In the zebrafish retina, Hh signals originating in the midline initiate retinal neurogenesis in the ventro-nasal patch of the neuroepithelium (Figure I-2) (Masai, Stemple et al. 2000). FGF is has been shown to act upstream of Hh signaling to control the initiation of retinal differentiation (Martinez-Morales, Del Bene et al. 2005). In zebrafish, perturbations of FGF signaling in zebrafish resulted in corresponding changes

in the expression of a transgenic reporter that is driven by Shh regulatory elements and supported the cooperation of the FGF and Hh pathways in the initiation and propagation of retinal differentiation (Vinothkumar, Rastegar et al. 2008). *Shh* and *tiggy-winkle hedgehog* (*twhh*), secreted by the first born RGC neurons, promote the further propagation of RGC differentiation (Neumann and Nuesslein-Volhard 2000). A second, temporally overlapping wave in the INL has also been shown to be Hh dependent and results in the production of amacrine cells, which in turn express Hh ligands that promote additional differentiation in the INL and ONL (Shkumatava, Fischer et al. 2004). The propagation of photoreceptor differentiation in the ONL is also thought to be Hh dependent and Hh signals originating from the RPE have been implicated to control this process (Stenkamp and Frey 2003). Hh signaling originating from differentiated retinal neurons is thought to also promote the proliferation of undifferentiated progenitors. In zebrafish, differentiated RGCs continue to express Hh ligands into adulthood (Neumann and Nuesslein-Volhard 2000), while undifferentiated retinal progenitors express the Hh receptor *patched2* throughout retinal development, making them competent to respond to Hh signaling (Stenkamp, Frey et al. 2000). These expression patterns within the retina are mostly conserved among vertebrate species (Wallace 2008).

Multiple studies have highlighted an apparent contradiction regarding the roles of the Hh pathway during retinal neurogenesis in different model organisms. Studies in zebrafish have mostly supported the role of Hh in promoting retinal differentiation at the expense of proliferation, as loss of Hh function results in a prolonged period of proliferation and defective differentiation (Stenkamp, Frey et al. 2002; Shkumatava and Neumann 2005). In addition, increased activity of PKA, a Hh pathway inhibitor, resulted in increased proliferation, while decreased PKA activity reduced proliferation (Masai,

Yamaguchi et al. 2005). In rodents, however, recombinant Shh-N promoted retinal progenitor cell proliferation in vitro (Jensen and Wallace 1997), and loss of Shh function results in decreased proliferation (Wang, Dakubo et al. 2005).

The roles of Hh in controlling both proliferation and differentiation in the vertebrate retina might be due to separate mechanisms that control these seemingly opposite processes. However, differentiation and cell fate decision have been shown to be tightly coupled to the regulation of cell cycle dynamics (reviewed in (Dyer and Cepko 2001). For instance, misexpression of the cell cycle inhibitor $p27^{xic1}$ in Xenopus results in early cell cycle exit and consequently in the over-production of early-born cell types (RGCs). Conversely, misexpression of Cyclin E1, a cell-cycle promoter, results in a bias towards later-born cell types, suggesting that the regulation of cell-cycle dynamics in the retina is important in cell fate decisions and in producing retinal tissue containing the correct ratio of neurons and glia (Ohnuma, Hopper et al. 2002). Interestingly, detailed analysis of cell cycle dynamics in both *Xenopus* and zebrafish suggested that the mitogenic role of Hh in the retina might be tightly linked to its function in promoting cell cycle exit and differentiation. In this study, over-expression of Hh reduced the length of the cell cycle and promoted proliferation while also resulting in early cell cycle exit. The shorter cell cycle was a result in the relative shortening of the G1 and G2 phases of the cell cycle and were also accompanied by the up-regulation of *cyclin2A, cyclinB1, cyclinD1, and cdc25* (Locker, Agathocleous et al. 2006). While these findings clearly show that cell cycle progression and cell cycle exit are tightly linked and are influenced by Hh activity, how these effects of the Hh pathway on retinal progenitors might affect cell fate decision is still unclear.

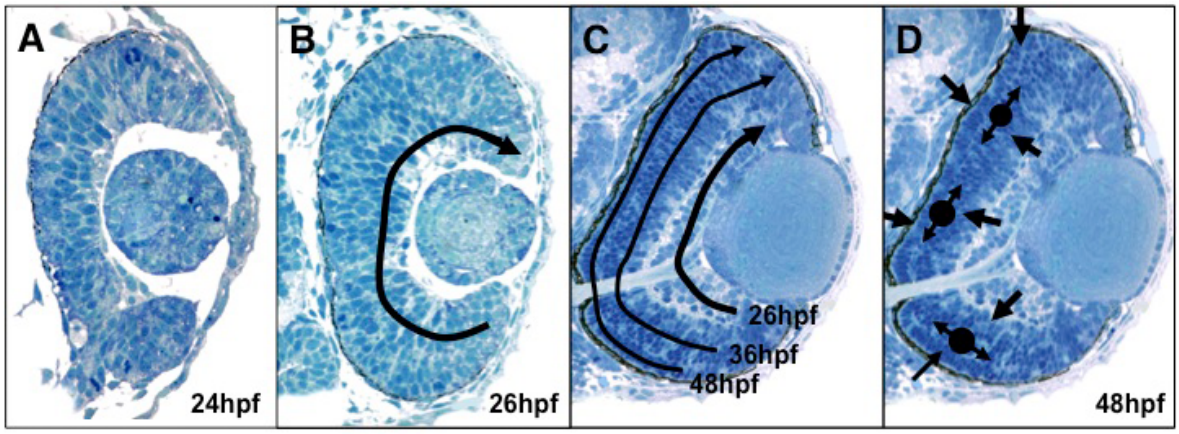

Figure I-2. Differentiation of the embryonic zebrafish retina. In zebrafish, differentiation of the proliferative retinal neuroepithelium (A) begins at 26 hpf (B) as a differentiation wave that is triggered by signals originating in the embryonic midline (arrow in B). Differentiation of the innermost cell layer, the GCL, occurs first, followed by temporally overlapping waves in the INL and ONL (arrows in C). In addition of midline signals, other retinal and extra-retinal signals have been shown to influence retinal differentiation (arrows in D).

I.3 RETINAL STEM CELLS IN GROWTH, REGENERATION AND DISEASE

The study of stem cells has been of major interest due to the growing potential of their use in therapeutic and regenerative medicine. The ability of zebrafish to regenerate various tissues after injury has attracted major attention from researchers seeking to identify the cellular and molecular mechanisms that enable this regenerative capacity, which is often absent in mammals (Johnson and Weston 1995; Vihtelic and Hyde 2000; Poss, Wilson et al. 2002). In the vertebrate retina, two populations of stem/progenitor cells have been identified: the Müller glia of the central retina, and the ciliary marginal zone (CMZ) at the most peripheral edge of the retina. Both of these stem cell populations add new neurons and glia to the zebrafish retina throughout the lifetime of the animal. Müller glia give rise to rod photoreceptor precursors that migrate along Müller glial processes to the photoreceptor layer. All other retinal cell types are continually produced in the post-embryonic CMZ.

I.3.1 The stem/progenitor cells of the CMZ

The spatial organization of the CMZ is thought to mirror and temporal sequence of early retinal developmental events. Retinal stem cells are thought to reside at the most peripheral edge of the CMZ, followed by proliferating progenitors more centrally, and finally differentiating progenitors (Figure I-3). At least some of the transcription factors

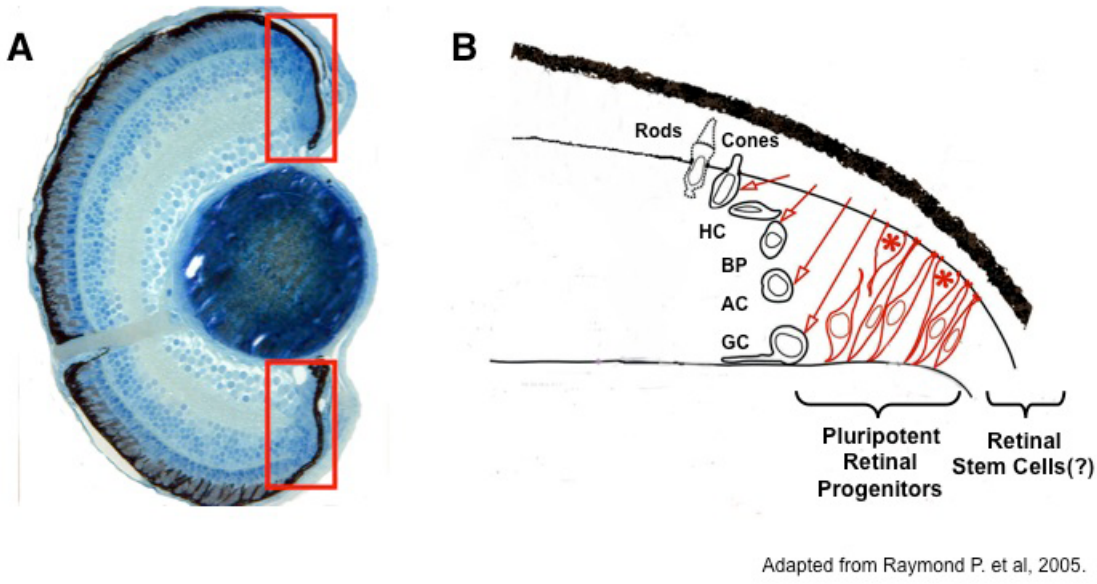

Figure I-3. The progenitor/stem cells of the retinal CMZ. The retinal CMZ is located at the retinal peripheral (A) and continues to proliferate throughout the lifetime of the animal, contributing to the growth of the retina. The spatial sequence of cells in the CMZ, with retinal stem cells at the most peripheral regions followed by proliferative progenitors and finally differentiating progenitors more centrally, is thought to recapitulate the temporal sequence of early retinal development (B).

known to be expressed in retinal progenitors during early retinal development are also expressed at the peripheral CMZ, such as *Chx10, Rx, Pax6* and *Six3* ((Perron, Kanekar et al. 1998; Raymond, Barthel et al. 2006), and are thought to promote proliferation and growth of retinal tissue throughout the lifetime of the animal. While the presence and identity of CMZ stem cells is still controversial, lineage tracing experiments performed in *Xenopus* showed that single-labeled cells can give rise to all retinal cell types, as well as RPE cells, suggesting that a population of stem cells exists at the retinal margin (Wetts, Serbedzija et al. 1989). Supporting this idea are phenotypes of zebrafish mutants in which a reduction in the size of the CMZ was accompanied by an expansion of the peripheral RPE (Gross, Perkins et al. 2005; Wehman, Staub et al. 2005).

The CMZ also possess regenerative capabilities, as lesions in the peripheral neural retina result in increased proliferation at the CMZ (Moshiri, Close et al. 2004). In chick, the maintenance of CMZ progenitors and CMZ-dependent retinal regeneration after retinotectomy is controlled by the concerted activity of the FGF, Hh, and BMP pathways (Spence, Madhavan et al. 2004; Spence, Aycinena et al. 2007). In mammals, however, regeneration from the retinal margin is not thought to normally occur (Locker, Borday et al. 2009). In a recent study, however, induced injury of RGCs resulted in limited proliferation at the mouse retinal margin and the expression of neuronal markers (Nickerson, Emsley et al. 2007). In addition, adult *Ptch1*$^{+/-}$ mice, possess persistent progenitors at the retinal margin that are capable of limited regeneration (Moshiri and Reh 2004). These results suggest that although stem cell at the mammalian retinal

margin, unlike those in lower vertebrates, are quiescent during normal development, they do possess neurogenic potential.

I.3.2 Müller glia

Neuroprotection, maintenance of retinal homeostasis, and the establishment of proper retinal lamination are some of the many functions attributed to Müller glia in the healthy retina (reviewed in (Bringmann, Pannicke et al. 2006)). In response to retinal disease or damage, Müller glia can become 'reactive', as characterized by changes in gene expression that are often followed by de-differentiation and proliferation. Virtually every human retinal disease is associated to some degree with Müller glial reactivity (Bringmann, Pannicke et al. 2006). In some cases, such as diabetic and proliferative retinopathies, and retinal detachment, Müller glia are thought to become reactive in response to primary detects arising in another cell type. In others, Müller glia are thought to be the primary cell type affected. Still, in many human eye diseases, the role of Müller glia is the onset of the disease is unclear.

Müller glia have long been known to possess neurogenic potential, having an intrinsic ability to give rise to newborn neurons. In the adult retina of lower vertebrates, differentiated Müller glia continue to express molecular markers that are characteristic of early retinal progenitors, such as pax6 and rx1 (Raymond, Barthel et al. 2006). Clusters of proliferating rod progenitors are closely associated with Müller glia and appear to

migrate along their processes, which span the apical-basal width of the adult retina, to their final location in the photoreceptor layer (Raymond and Rivlin 1987; Julian, Ennis et al. 1998). A recent study utilizing transgenic zebrafish that express GFP under the control of a Müller glia-specific promoter (*Tg(gfap:GFP)*) as a lineage tracer has shown that proliferating rod progenitors retain low levels of GFP, suggesting that Müller glia indeed give rise to rod photoreceptor precursors in the adult retina (Bernardos, Barthel et al. 2007). These findings support the neurogenic potential of Müller glia and raise the intriguing possibility of using Müller glia as a source for cell replacement therapies for humans suffering from age-related photoreceptor loss, for example.

While in the uninjured adult retina Müller glia give rise strictly to progenitors destined to the rod lineage, Müller glia are also able to dedifferentiate, proliferate, and replenish all neuronal cell types in response to retinal damage (Cameron 2000; Bernardos, Barthel et al. 2007). While conclusive lineage tracing data to show Müller glia as the sole source of regenerated neurons have not yet been published, the use of zebrafish transgenic lines together with extensive immuno-histochemical analysis has strongly supported the role of Müller glia as the stem cell population responsible for the regenerative response in the central retina (Fausett and Goldman 2006; Bernardos, Barthel et al. 2007). Since mutant analysis in adults is labor intensive and time consuming, multiple labs have also performed microarray analyses after retinal damage in order identify genes that play a role in these processes. Consistent with the idea that regenerative processes often re-capitulate embryonic development, these studies

identified known developmental pathways such as BMP, Notch, Wnt and Hedgehog as being transcriptionally regulated in reactive Müller glia (Kassen, Ramanan et al. 2007; Qin, Barthel et al. 2009). It remains to be seen what exact roles of each of these pathways in retinal regeneration, and whether the genetic and/or chemical manipulation of these pathways might have therapeutic value in humans.

I.3.3 The Hh pathway in the adult retina

In addition to its developmental roles, the Hh pathway functions in the adult CNS to maintain neural stem cell populations (Fuccillo, Joyner et al. 2006). In the adult forebrain, Hh signaling has been shown to function in two regions of continual neurogenesis. In subventricular zone (SVZ) of the lateral ventricles and the subgranular zone (SGZ) of the dentate gyrus within the hippocampus, Shh controls the number of proliferating progenitors (Lai, Kaspar et al. 2003; Machold, Hayashi et al. 2003). In Shh null mice, SVZ-derived olfactory bulb and SGZ-derived hippocampal granule cells are depleted. *In vivo* analysis of Shh-dependent control of progenitor proliferation in these regions is control through the regulation of self-renewal properties of slow-cycling neural stem cells. In this context, Shh acts to promote proliferation of stem cells in late embryonic stages and further maintains this stem cell niche into adulthood (Ahn and Joyner 2005). These findings highlight an essential role for Hh signaling in maintaining the neurogenic potential of adult stem cell niches in the CNS.

In the adult mouse retina, Hh ligand expression has been reported in RGCs and the RPE and *Ptch* is expressed in Müller glia and the ciliary margin (Jensen and Wallace

1997; Takabatake, Ogawa et al. 1997; Moshiri and Reh 2004). Interestingly, the expression of *Ptch* in the known stem/progenitor cell populations of the adult retina suggests that the Hh pathway might function to control some aspects of post-embryonic retinal neurogenesis. Indeed, in the post-hatch chick, Shh promotes the proliferation of progenitors located in the CMZ-like retinal margin (Moshiri, McGuire et al. 2005). In *Ptch1*+/- post-natal mice a population of proliferative progenitors that resembles the CMZ found in lower vertebrates, but that is normally absent in mammals, has been reported at the retinal margin (Moshiri and Reh 2004). The functional significance of Hh activity in Müller glia has yet to be established *in vivo*. *In vitro*, rat Müller glia de-differentiated, proliferated, and expressed rod photoreceptor markers upon exposure to recombinant Shh, suggesting that Hh pathway activity might indeed act to control the neurogenic potential of Müller glia (Wan, Zheng et al. 2007). Müller glia are also known to promote proper retinal organization and lamination (see section I-3). Inhibition of Hh signaling via exposure to an anti-Shh antibody disrupted lamination in rat retinal explants and administration of Shh to these explants restored proper Müller glia morphology and lamination (Wang, Dakubo et al. 2002), suggesting a role for Hh in promoting normal retinal structure.

In a recent study, human embryonic stem cells (hESCs) expressed the retinal progenitor markers Six3, Pax6, Chx10 and Rx, and subsequently differentiated into retinal neurons upon exposure to Shh. In addition, when transplanted into the subretinal space of visually-impaired albino rabbits, these progenitors were able to incorporate into the retina and partially restore visual function (Amirpour, Karamali et al. 2011). These results not only illustrate the ability of Hh activity to influence retinal stem cell identify and maturation, but also highlights the potential of Shh as a potential factor for use in

stem cell therapies in medical treatments of certain types of blindness, such as retinal degenerations. *In vivo* studies utilizing *Ptch1*$^{+/-}$ mice have supported the therapeutic potential of the Hh pathway. Breeding of *Ptch1*$^{+/-}$ mice into a retinal degeneration background (pro23his rhodopsin transgenic) resulted in the production of newly generated photoreceptors, suggesting that the over-active Hh pathway in these mutants has activated neurogenesis (Moshiri and Reh 2004).

A study analyzing retinal morphology in adult zebrafish with a loss-of-function mutation in *Shh* (*syu*$^{+/-}$) revealed cone photoreceptor abnormalities characterized by localized photoreceptor loss and morphological abnormalities (Stenkamp, Satterfield et al. 2008). In humans, photoreceptor degenerations are the most common form of blindness in the Western world and involve the loss of vision due to dystrophy and/or death of retinal photoreceptors. These pathologies can be roughly divided into those conditions that initially affect rod photoreceptors, such as retinitis pigmentosa (RP), and those that initially affect cone photoreceptors, such as macular degeneration (Goldsmith and Harris 2003). In mature photoreceptors, proteins that are required for growth and maintenance of photoreceptor OS's, as well as for phototransduction, are transported along polarized microtubules from the basal IS to the apical OS. Proper development and subsequent maintenance of vertebrate photoreceptors relies on the establishment of proper apical–basal polarity, as well as the function of the transport machinery. It is therefore not surprising that of the more than 100 genetic loci known to cause photoreceptor degenerations in vertebrates, most affect the structure and function of the OS (Hartong, Berson et al. 2006). In addition, photoreceptor degenerations can also be caused indirectly by primary defects in the RPE, which serves an important function in photoreceptor health and homeostasis. Considering that Hh ligands expressed in the RPE

during zebrafish embryonic development have been suggested to influence proper photoreceptor development (Stenkamp and Frey 2003), it is possible that disrupted Hh signaling in *syu*$^{+/-}$ mutant RPE might underlie cone abnormalities in these mutants.

While the studies discussed above begin to indicate roles of the Hh pathway in the adult retina, our understanding of the process is still limited. Analysis of mutations in Hh components is invaluable in order to provide models to determine the requirement and function of the Hh pathway during retinal homeostasis and disease. Indeed, the mouse *Ptch1*$^{+/-}$ mutant has been established as a model for the study of BCNS, a congenital disorder that includes ocular abnormalities (see chapter III) (Black, Mazerolle et al. 2003), and additional studies utilizing the zebrafish *syu*$^{+/-}$ mutant line may further contribute to our understanding of the molecular mechanisms underlying Hh function in photoreceptor homeostasis. Furthermore, the continual development of conditional gene knockdown strategies will greatly facilitate efforts to investigate the roles of specific genetic pathways, such as Hh, in the adult retina.

The work presented here addresses open questions regarding the roles of the Hh pathway during both embryonic development and in adult retinal tissues through the study of the zebrafish *ptc2* mutant line. My research sheds new light on Ptc-dependent Hh signaling in the vertebrate retina and contributes to our understanding of the roles of Ptc in controlling proliferation and proper patterning and structure in the retina. In this work, I have also established the *ptc2* mutant line as a model for the study of BCNS and provided important insight into the relationship between retinal over-proliferation and BCNS-related ocular pathologies.

CHAPTER II

Expanded progenitor populations, vitreo-retinal abnormalities, and Müller glia reactivity in the *ptc2* retina

II.1 INTRODUCTION

During retinal development, proliferation and differentiation must be tightly coordinated in order to produce a tissue of the proper size and containing the correct cell types (Neumann 2005). The Hh pathway has been shown to play a critical role in controlling these two seemingly opposite processes (Marti and Bovolenta 2002). Early in retinal development, the optic vesicle is composed of a population of proliferating neuroepithelial cells that will ultimately give rise to the mature retina (Li, Hu et al. 2000). Differentiation of the retinal neuroepithelium occurs in a succession of temporally overlapping waves (Amato, Boy et al. 2004). In the zebrafish, the first cells to exit the cell cycle become retinal ganglion cells (RGCs), which differentiate in a Sonic Hh (Shh)-dependent wave (Neumann and Nuesslein-Volhard 2000). A second Hh-dependent wave of differentiation in the inner nuclear layer (INL) occurs almost simultaneously with the first wave, and is responsible for the differentiation of the multiple cell types of the INL (horizontal, amacrine, and bipolar cells, and Müller glia) and the rod and cone photoreceptors of the outer nuclear layer (ONL) (Shkumatava, Fischer et al. 2004). In addition, extra-retinal Hh signaling originating in the retinal pigment epithelium (RPE)

has been suggested to direct photoreceptor differentiation (Stenkamp and Frey 2003). While the role of the Hh pathway in cell cycle exit and differentiation of retinal progenitors is well described, comparatively less is known about its possible influence on cell fate decisions. In *Xenopus*, over-expression of p27Xic1, which promotes cell cycle exit, results in increased numbers of early-born cell types (RGCs), while the over-expression of cyclin E1, which inhibits cell cycle exit, biases cell fate towards late-born cell types (e.g. Müller glia) (Ohnuma, Hopper et al. 2002). Similarly, Shh has been shown to promote early cell cycle exit in the *Xenopus* retina (Locker, Agathocleous et al. 2006); however, a direct role of the Hh pathway in dictating retinal cell fate has yet to be established *in vivo*.

While the cells of the central retina of the zebrafish exit the cell cycle by 60 hours post fertilization (hpf) (Schmitt and Dowling 1999), a population of retinal progenitors at the CMZ is maintained and continues to proliferate throughout the animal's lifetime (Wetts, Serbedzija et al. 1989; Raymond, Barthel et al. 2006). The spatial pattern of cells within the CMZ, with retinal stem cells at the most peripheral region followed by proliferative retinal progenitors and finally differentiating progenitors more centrally, is thought to recapitulate the temporal sequence of early retinal development (Perron, Kanekar et al. 1998). Some of the factors that control early retinal development, such as *notch, rx1, pax6a, and vsx2/chx10*, are expressed in the zebrafish CMZ (Raymond, Barthel et al. 2006). In *Xenopus*, expression of *gli2*, *gli3*, and *smoothened*, is found at the retinal margin, suggesting a role for the Hh pathway in the stem cell/progenitor

population in the CMZ (Perron, Boy et al. 2003). Shh over-expression studies in chick support a role for the Hh pathway as a mitogen in the CMZ (Moshiri, McGuire et al. 2005), consistent with its described mitogenic effects on retinal progenitors in early zebrafish and mouse retinal development (Jensen and Wallace 1997; Stenkamp, Frey et al. 2002).

Invaluable knowledge regarding Hh function in the developing retina has been gained from the analysis of Hh pathway mutants in zebrafish. For example, the zebrafish mutants *syu* (*shh*) and *smu* (*smoothened*) have helped elucidate the complex mechanisms of Hh-dependent neural differentiation and proliferation (Stenkamp, Frey et al. 2002; Stenkamp and Frey 2003). However, retinal differentiation is severely defective or altogether absent in these mutants due to defective Hh signaling, making it difficult to arrive at definitive conclusions about the possible role of the Hh pathway in cell fate decisions. To address this issue, we analyzed retinal development in the zebrafish *ptc2*$^{-/-}$ mutant, in which the Hh pathway is 'over-active'. *ptc2*$^{-/-}$ mutants possess a non-sense mutation in the exon encoding the sixth trans-membrane domain of Patched2 (Koudijs, den Broeder et al. 2005), which is predicted to abolish its function (Johnson, Milenkovic et al. 2000; Koudijs, den Broeder et al. 2005). Loss of Patched function results in an 'over-active' Hh pathway due de-repression of Smoothened (Goodrich, Milenkovic et al. 1997). Normally, in the absence of Hh ligand, the Patched protein inhibits the activity of Smoothened. Binding of the Hh ligand to its Patched receptor relieves this inhibition, ultimately resulting in increased transcription of Hh target genes (Murone, Rosenthal et

al. 1999; Taipale, Cooper et al. 2002). A non-functional Patched would therefore result in loss of inhibition on, and constitutive activity of, Smoothened in *ptc2* deficient cells. *ptc2*$^{-/-}$ mutants possess increased proliferation in multiple tissues including the retinal CMZ (Koudijs, den Broeder et al. 2005). Utilizing this mutant as a tool, we sought to gain further insight into Hh function with respect to retinal cell fate decisions and to determine how Hh activity influences progenitor proliferation at the CMZ. Our results revealed no significant change in neuronal composition within the retina, but we did note a reduction in differentiated Müller glia that was accompanied by localized Müller glial reactivity and abnormalities at the vitreo-retinal interface. In addition, the results of cell cycle analyses suggest that the over-proliferation at the CMZ likely results from an expansion of the progenitor cell population, and not from direct effects of the mutation on cell cycle kinetics within progenitor cells.

II.2 RESULTS

II.2.1 *ptc2*$^{-/-}$ mutants possess enlarged retinas and overgrown irises, while lens and RPE morphology are largely normal

ptc2$^{-/-}$ mutants were isolated based on expanded PCNA staining in multiple tissues including the retinal CMZ (Koudijs, den Broeder et al. 2005). While mutant embryos appear to be of normal size at 5 days post fertilization (dpf) (Figure II-1A), their retinas are enlarged (Figure II-1D,E) and they possess reduced pupil size ((Koudijs, den Broeder

et al. 2005) and Figure II-1B,C). To characterize ocular tissues in *ptc2*$^{-/-}$ mutants, we performed histological analyses at 33hpf, around the time of the onset of retinal differentiation, and at 5dpf, when the laminar organization of the embryonic retina is complete (Schmitt and Dowling 1999; Gross, Perkins et al. 2005). *ptc2*$^{-/-}$ retinas appear largely normal at 33dpf and do not possess increases in retinal cell number relative to phenotypically wild-type siblings (Figure II-2A,B,E). At 5dpf, however, *ptc2*$^{-/-}$ retinal cells are more tightly packed and mutant retinas contain a larger number of cells as compared to their phenotypically wild-type siblings (Figure II-2C,D,E). Normal retinal lamination of differentiated neurons has been shown to be dependent on Shh signaling *in vitro* (Wang, Dakubo et al. 2002). Lamination is largely normal in *ptc2*$^{-/-}$ mutants and all major cell types are present (Figure II-2C,D). Loss-of-function experiments in both frog and mouse have suggested a role for the Hh pathway in the development of the RPE (Perron, Boy et al. 2003; Dakubo, Mazerolle et al. 2008). In *ptc2*$^{-/-}$ mutants the RPE is of normal thickness and morphology. The Hh pathway has also been implicated in lens development (Karlstrom, Talbot et al. 1999; Swindell, Zilinski et al. 2008), and the reduced pupil size present in *ptc2*$^{-/-}$ mutants was previously attributed to potential degeneration of the lens (Heisenberg, Brand et al. 1996). No gross morphological defects are evident in the lenses of *ptc2*$^{-/-}$ mutants at either 33hpf or 5dpf, however. Additionally, histological analyses revealed an abnormality in the anterior segment of *ptc2*$^{-/-}$ eyes where the iris was over-grown and extended further over the lens than in sibling retinas (Figure II-2D). The enlarged iris and the overgrowth of the CMZ are the likely causes of the decreased pupil size, rather than defects in lens morphology.

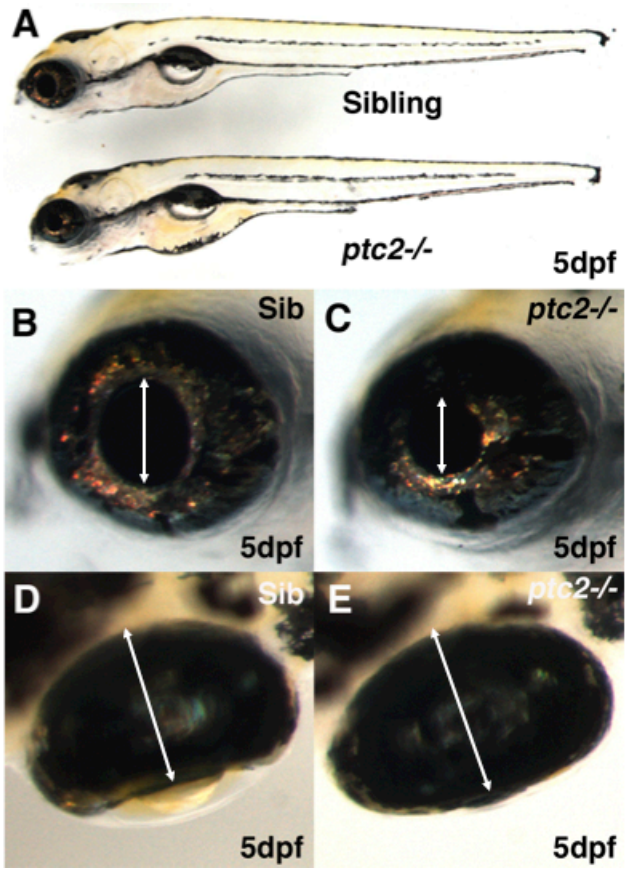

Figure II-1. ***ptc2*$^{-/-}$ mutants possess enlarged retinas and reduced pupil size**. Whole embryo lateral view shows that *ptc2*$^{-/-}$ embryos are largely normal compared to their siblings (A). High magnification images of sibling (B) and *ptc2*$^{-/-}$ (C) eyes reveal reduced pupil size in *ptc2*$^{-/-}$ (arrows in B,C). Dorsal view images illustrate the overgrowth of retinal tissue as the retina is thicker and appears to engulf the lens (arrows in D, E).

II.2.2 Neuronal composition of the *ptc2*$^{-/-}$ retina is normal, while Müller glia are under-represented

Hh signaling has been shown to be required for the differentiation of multiple cell types in the vertebrate retina (Zhang and Yang 2001; Stenkamp and Frey 2003; Shkumatava, Fischer et al. 2004) and is thought to promote cell fate decisions by modulating the timing of cell cycle exit (Wallace 2008). Shh gain-of-function experiments in *Xenopus* link a faster cell cycle with early cell cycle exit (Locker, Agathocleous et al. 2006), which in turn results in an over-production of early-born retinal cell types at the expense of late-born cell types (Ohnuma, Hopper et al. 2002). We therefore reasoned that in *ptc2*$^{-/-}$ retinas, where the Hh pathway is over-active, early-born cell types would be over-represented while late-born cell types would be reduced in number. Cell counts from histological sections of *ptc2*$^{-/-}$ mutants revealed a statistically significant increase in cell number in all three retinal nuclear layers (GCL, INL, and ONL), as well as in total retinal cells, when compared to those from phenotypically wild-type siblings (Figure II-2E). However, no significant change in the proportion of each layer was observed when the number of cells per layer was calculated as a percentage of total retinal cells (Figure II-2F). This suggests that all three retinal cell layers are proportionally increased in *ptc2*$^{-/-}$ retinas.

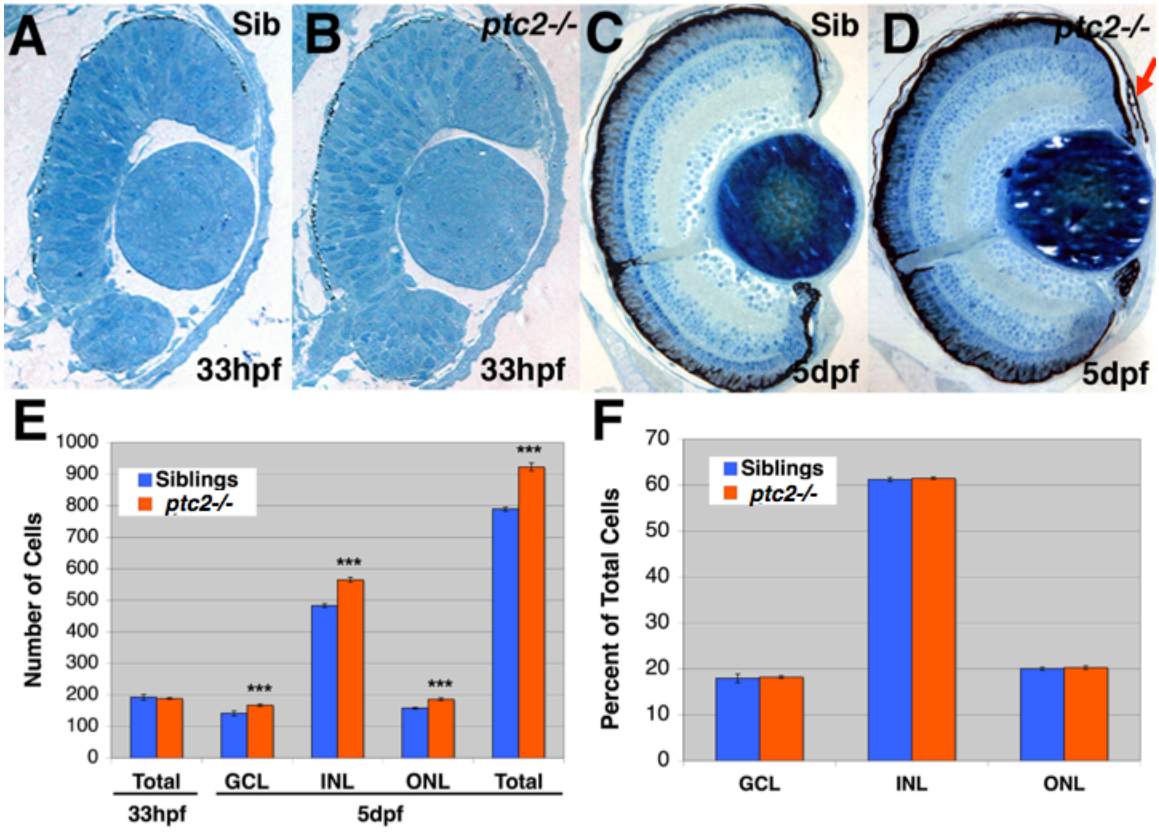

Figure II-2. *ptc2*$^{-/-}$ mutant retinas possess proportional increases in cell numbers in all three nuclear layers. Histology of sibling (A,C) and *ptc2*$^{-/-}$ mutant (B,D) retinas at 33hpf (A,B) and 5dpf (C,D). E) Quantification of total number of retinal cells at 33hpf and 5dpf, and of cells in each nuclear layer at 5dpf (18.0%, 17.0%, and 17.8% increases in GCL, INL, and ONL, respectively. $^{***}p<0.0001$). F) Graph of the proportion of each nuclear layer as a percentage of total retinal cells at 5dpf. Scale Bars = 20um in A,B and 100um in C,D.

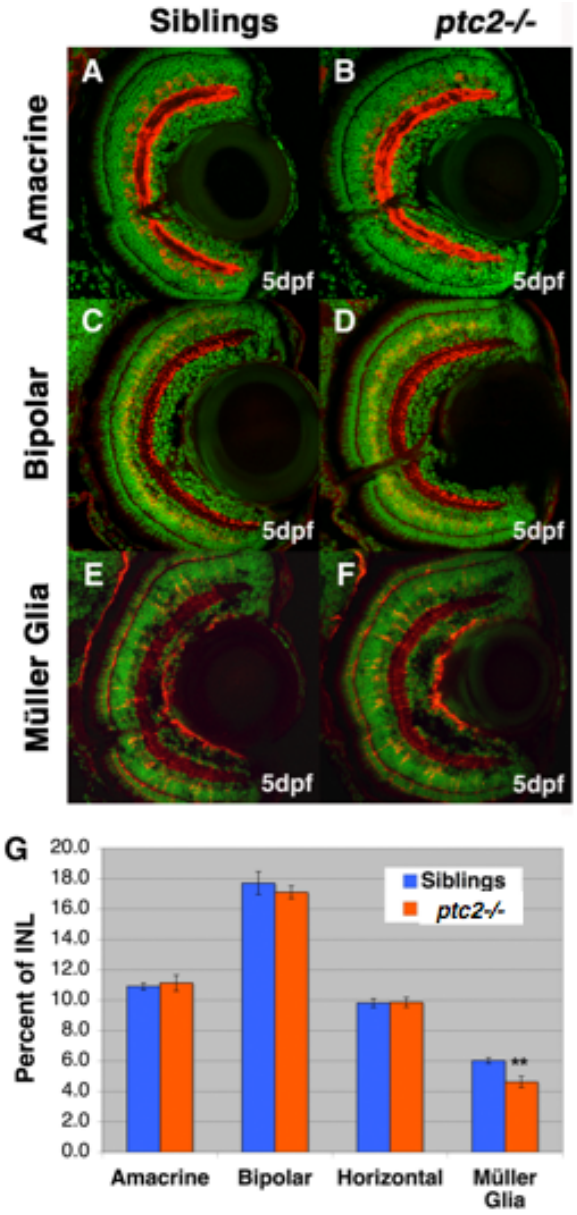

Figure II-3. All neuronal cell types in the *ptc2*$^{-/-}$ INL are increased proportionately, while Müller glia are reduced. Immunohistochemistry of sibling (A,C,E) and *ptc2*$^{-/-}$ (B,D,F) retinas for Amacrine cells (A,B), Bipolar cells (C,D), and Müller glia (E,F). G) Cell counts for each INL cell type. No significant change is cell number in all neuronal cell types when calculated as a percentage of total INL cells. Müller glia, however, were reduced in *ptc2*$^{-/-}$ retinas by 29% relative to sibling retinas (**p=0.0013).

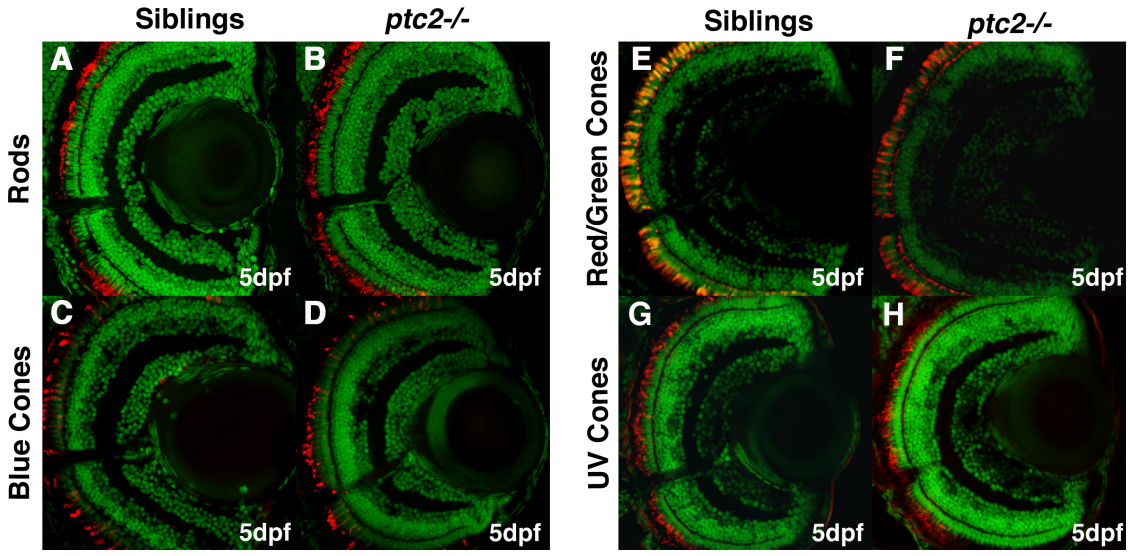

Figure II-4. Photoreceptors appear unaffected in *ptc2*$^{-/-}$ retinas.
Immunohistochemical analysis of rods (A,B), blue cones (C,D), red/green cones (E,F), and UV cones (G,I) in sibling (A,C,E,G) and *ptc2*$^{-/-}$ retinas (B,D,F,I). All photoreceptor types were present and appear normal.

It is possible that in *ptc2*$^{-/-}$ mutants, an overactive Hh pathway could alter neuronal composition within each layer. To test this hypothesis, immunohistochemistry using markers for each specific cell type in the INL and ONL was performed on retinal sections at 5dpf, when all cell types are normally differentiated and the embryonic retina is fully developed. Since the GCL layer is almost exclusively composed of retinal ganglion cells (RGCs), no further analysis was performed on this layer. In order to analyze cell types within the INL, immunohistochemistry was performed using antibodies that mark amacrine cells (5E11), bipolar cells (PKC) and Müller glia (Glutamine Synthetase, GS), while horizontal cells were detected according to their stereotypical morphology in histological sections. This analysis revealed that while the proportion of each neuronal cell type in the INL was not significantly changed in *ptc2*$^{-/-}$ mutants, there was a statistically significant decrease in the proportion of differentiated Müller glia (Figure II-3). For the ONL, immunohistochemistry was performed to detect rods (Zpr3) and each three cone opsins (blue, red/green, and UV) (Vihtelic, Doro et al. 1999) . Due to the diffuse nature of the staining of some of the markers used, precise cell counts were not performed on these cell types. However, immunohistochemisty did show that these cell types are present in *ptc2*$^{-/-}$ and qualitatively appear unaffected (Figure II-4). In all, *ptc2*$^{-/-}$ mutants possess more cells in their retinas and cell types, except for Müller glia, were present at identical ratios to those observed in phenotypically wild-type siblings.

II.2.3 *ptc2*$^{-/-}$ mutants possess abnormalities at the vitreo-retinal boundary and localized Müller glial reactivity

Müller glial endfeet terminate at, and contribute to, the inner limiting membrane (ILM), which separates the retina from the vitreous (Gabriel, Wilhelm et al. 1993). Interestingly, our immunohistochemical analysis of differentiated Müller glia revealed disruptions in the integrity of the ILM (Figure II-5A,B). Since humans who possess mutations in the *PTCH* gene (orthologue of the zebrafish *ptc2* (Lewis, Concordet et al. 1999)) have been previously shown to possess abnormalities in the vitreo-retinal interface (Black, Mazerolle et al. 2003), we sought to further investigate this phenotype. High magnification examination of GS-stained retinas revealed disruptions in the integrity of the ILM in 40% of the *ptc2*$^{-/-}$ retinas examined. While Müller glial endfeet form a tight and continuous boundary at the vitreo-retinal boundary in sibling retinas, the ILM in *ptc2*$^{-/-}$ retinas was often discontinuous and disorganized, and endfeet did not appear to properly terminate in the ILM (Figure II-5A,B). In *Ptch1*$^{+/-}$ mice, ocular abnormalities are accompanied by the upregulation of GFAP, a marker of reactive Müller glia, and ectopic proliferation of Müller glia in the central retina (Black, Mazerolle et al. 2003). Immunohistochemical analysis of GFAP revealed that its levels in Müller glia were indeed higher in *ptc2*$^{-/-}$ mutant retinas as compared to their phenotypically wild-type siblings (Figure II-5C,C',D,D'). Higher levels of GFAP were often localized to Müller glial endfeet in the central retina, adjacent to the optic nerve. However, BrdU incorporation assays in *ptc2*$^{-/-}$ mutants at 5dpf did not reveal ectopic proliferation in the

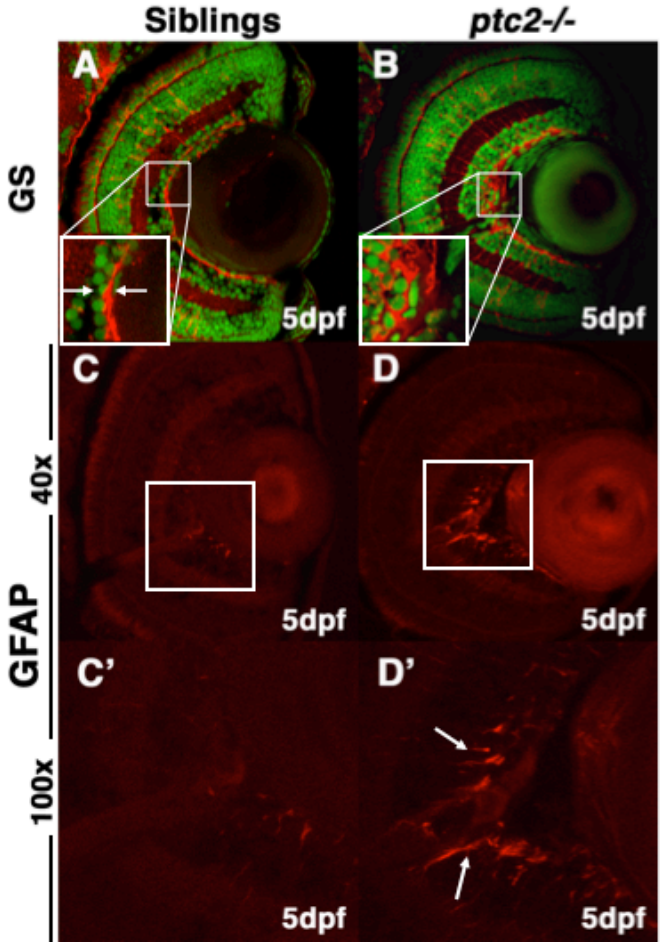

Figure II-5. *ptc2*$^{-/-}$ mutants display Müller glial reactivity and morphological abnormalities in the ILM. Immunohistochemical analysis using glutamine synthetase (GS) antibody, which marks differentiated Müller glia and their endfeet at the ILM, highlights disruptions in the ILM. (A) In sibling retinas, the ILM is tight and continuous (inset). (B) In *ptc2*$^{-/-}$ retinas the ILM is discontinuous and Müller glial endfeet do not terminate properly at the ILM (inset). Glial fibrillary acidic protein (GFAP) antibody staining reveals elevated immuno-reactivity in the inner retina, adjacent to the optic nerve of *ptc2*$^{-/-}$ (D and arrows in D') mutant retinas as compared to siblings (C, C'). Approximately 40% of analyzed mutants displayed significant ILM disruptions and elevated GFAP levels.

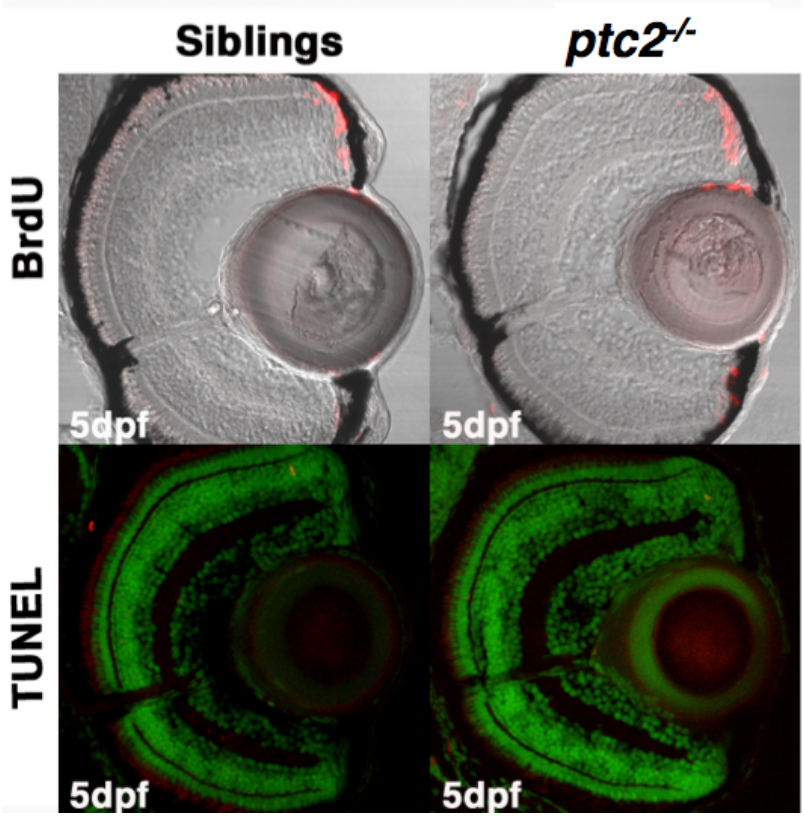

Figure II-6. *ptc2*$^{-/-}$ mutants do not possess ectopic cell death or proliferation in the central retina and/or optic stalk.
(A,B) BrdU incorporation and (C,D) TUNEL assays in 5dpf (A,C) phenotypically wild-type siblings and (B,D) *ptc2*$^{-/-}$ mutants.

central retina (Figure II-6), suggesting that the upregulation of GFAP is not coupled with Müller glial proliferation in this context. In addition, the localized nature of Müller glial reactivity in not due to abnormal cell death in the *ptc2*$^{-/-}$ retina and/or optic nerve since no apoptotic nuclei were detected by TUNEL assays (Figure II-6).

II.2.4 *ptc2* is expressed in the progenitor/stem cell populations of the CMZ

We next sought to better understand the nature of the over-proliferation at the CMZ by defining the retinal cell populations that express the *ptc2* transcript. At 56hpf, prior to the time that the overgrowth phenotype becomes detectable, *ptc2* expression was visible at the retinal margin, corresponding to the undifferentiated population of retinal progenitors of the CMZ (Figure II-7A). Expression continued to be restricted to the CMZ at 72hpf (Figure II-7B), consistent with previously reported expression of Hh target genes in the *Xenopus* CMZ (Perron, Boy et al. 2003). Closer inspection of *ptc2* expression in the CMZ at 72hpf revealed that low-level *ptc2* expression was detected throughout most of the CMZ, consistent with previous reports (Stenkamp, Frey et al. 2000). Staining was more robust in two distinct CMZ cell populations; the first was immediately apposed to the RPE and the second was a patch of cells at the edge of the CMZ, bordering the differentiated GCL (arrows in Figure II-7E). Hh ligands have been shown to be expressed in both the RPE (Perron, Boy et al. 2003) and the GCL (Neumann and Nuesslein-Volhard 2000; Wang, Dakubo et al. 2002) during retinal development, and Patched genes are expressed in a Hh-dependent manner (Lewis, Concordet et al. 1999).

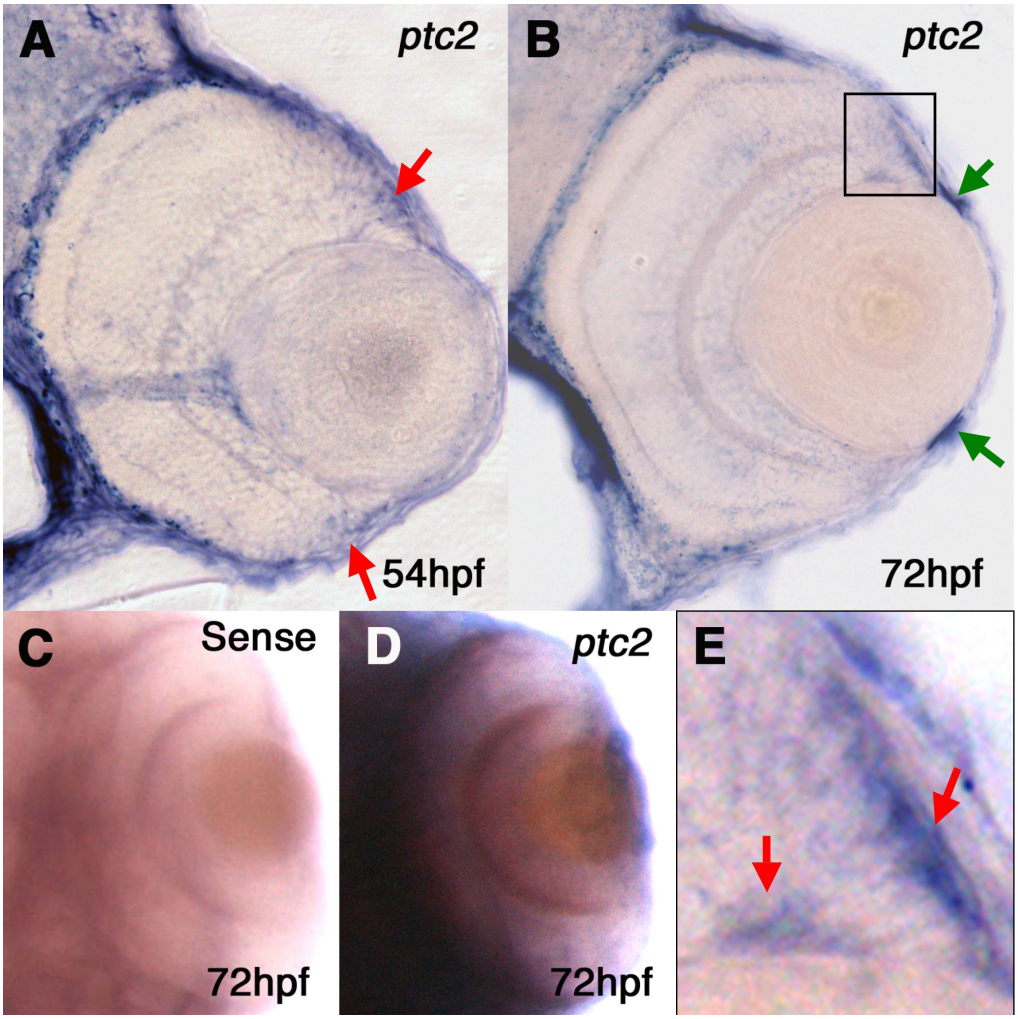

Figure II-7. *ptc2* is expressed at the zebrafish CMZ. *In-situ* hybridization for the *ptc2* transcript shows expression at the retinal margin at 56hpf (arrows in A). At 72hpf, transcript is detected throughout the CMZ (B) and at the iridio-corneal angle (green arrows), with high magnification of CMZ region (E) revealing more robust staining adjacent to the RPE and in a region just apposed to the GCL (red arrows). Whole mount images from a dorsal view of sense (C) and anti-sense (D) probes *in-situ* hybridization show that staining is specific.

Thus, these CMZ regions may reflect areas of active Hh signaling. Finally, the *ptc2* transcript is expressed at the iridio-corneal angle, where the iris and cornea converge (Soules and Link 2005) (arrows in Figure II-7B).

II.2.5 The number of proliferating progenitors, but not the rate of progenitor proliferation, is increased in the *ptc2*$^{-/-}$ CMZ

Ptc1$^{+/-}$ mice possess increased numbers of progenitor cells at all stages of retinal development, suggesting a role for the Hh pathway in controlling the size of retinal progenitor populations in the ciliary margin, and these mice also possess a persistent progenitor population in the retinal margin as adults (Moshiri and Reh 2004). Studies in post-hatch chicks suggest that Shh regulates the proliferation of CMZ progenitors (Moshiri, McGuire et al. 2005). In addition, Shh over-expression studies in *Xenopus* have revealed a role for the Hh pathway in regulating the length of the cell cycle in the progenitor populations of the CMZ (Locker, Agathocleous et al. 2006). Given the differing roles for Hh pathway activity in these contexts, we wanted to determine whether the over-proliferation in *ptc2*$^{-/-}$ retinas was due to an increase in the number of proliferating cells and/or an increase in the proliferation rate of the progenitor cell population in the CMZ. To address these possibilities, we first performed a 'pulse-chase' BrdU analysis on *ptc2*$^{-/-}$ and sibling embryos starting at 64hpf, when a mild retinal phenotype is first detectable. Embryos were sorted according to phenotype and exposed to a short pulse (15min) of BrdU. Half of the embryos were immediately fixed for

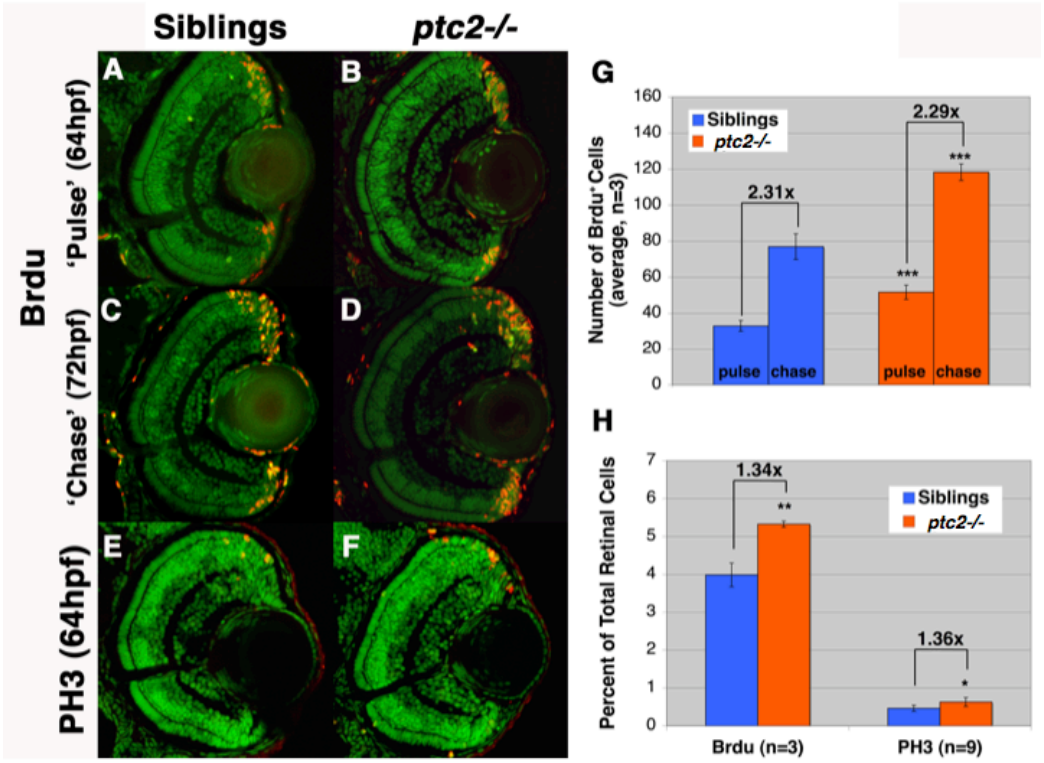

Figure II-8. The number of proliferating CMZ progenitors is increased in *ptc2*$^{-/-}$, while proliferation rate is unaffected. (A,B) BrdU 'pulse' in sibling (A) and *ptc2*$^{-/-}$ mutants (B). BrdU^{+} cells are observed throughout the CMZ. (C,D) After an 8 hour 'chase', BrdU^{+} cells are observed at the CMZ, as well as within the retina of sibling (C) and *ptc2*$^{-/-}$ mutants (D). E,F) PH3 labeling in sibling (E) and *ptc2*$^{-/-}$ (F) at 64hpf. G) Quantification of BrdU^{+} cells in sibling and *ptc2*$^{-/-}$ after 'pulse-chase' experiment. *ptc2*$^{-/-}$ retinas contained 67% more BrdU^{+} cells than sibling retinas at 64hpf. After an 8 hour 'chase', the number of BrdU^{+} cells increased by almost identical ratios in siblings (2.31-fold increase) and *ptc2*$^{-/-}$ mutants (2.29-fold increase, $^{***}p<0.0001$). H) Quantification of BrdU- and PH3-positive cells as a percentage of total retinal cells in *ptc2*$^{-/-}$ and wild-type siblings at 64hpf. The proportion of both BrdU^{+} and PH3^{+} cells increased by similar ratios (1.34-fold increase, $^{**}p<0.001$ and 1.36-fold increase, $^{*}p<0.01$, respectively).

sectioning, while the other half were transferred to non-BrdU containing fish water and fixed 8 hours later (72hpf). This enabled us to determine the rate of progenitor proliferation in both *ptc2*$^{-/-}$ and sibling retinas during the 8 hour chase period. The results of these assays revealed that, on average, *ptc2*$^{-/-}$ retinal sections contained 67% more BrdU-positive cells in the CMZs when compared to phenotypically wild-type sibling retinas at 64hpf (Figure II-8A,B, E). At 72hpf, after the 8 hour 'chase', the average number of BrdU-positive cells in *ptc2*$^{-/-}$ and phenotypically wild-type sibling retinal sections increased by almost identical ratios (2.29-fold changes in *ptc2*$^{-/-}$ vs. 2.31 fold change in sibling sections, Figure II-8C,D, E). From these data, we can therefore infer that, on average, each proliferating cell gave rise to 2.3 daughter cells during the 8 hour 'chase' period. To further analyze proliferation, we assayed the expression of phosphohistone H3 (PH3) and these analyses revealed that the proportion of CMZ progenitors occupying the M phase of the cell cycle increased by approximately 34% in *ptc2*-/- retinas at 64hpf, when compared to their phenotypically wild-type siblings (Figure II-8E,F,H). The degree of this increase was almost identical to that observed in progenitor cells occupying the S phase using BrdU as a marker (Figure II-8A,B,H), suggesting that the length of the S and M phases was not changed relative to the other. these results support a model in which the expanded CMZ of *ptc2*$^{-/-}$ mutants arises from an expansion of the progenitor cell population therein, and not from changes in the length of the cell cycle in these progenitor cells.

II.3 DISCUSSION

II.3.1 Retinal patterning is normal in *ptc2*$^{-/-}$ mutants

Characterization of *ptc2*$^{-/-}$ retinas with respect to cell type composition revealed that over-activity of the Hh pathway did not affect retinal patterning. Cell count analysis of all major retinal neuronal cell types revealed no change in the proportion of each cell type. This finding was surprising in light of the well established role of the Hh pathway in patterning and cell fate decisions throughout the CNS in a number of model systems (Ekker, Ungar et al. 1995; Ericson, Rashbass et al. 1997; Goodrich, Milenkovic et al. 1997), as well as studies in *Xenopus* which show that the over-expression of Shh results in early cell cycle exit (Locker, Agathocleous et al. 2006), and that can, in turn, influence cell fate decisions (Ohnuma, Hopper et al. 2002). While mouse *Ptch1*$^{+/-}$ (the orthologue of the zebrafish *ptc2* (Lewis, Concordet et al. 1999)) mutants were found to possess no defects in retinal cell fate specification, *Ptch1*$^{-/-}$ mice could not be examined since they die *in utero* (Moshiri and Reh 2004). Our results indicate that Ptc2-dependent Hh signaling does not likely play an integral role in neuronal cell fate decisions in the zebrafish retina. These findings raise the possibility that the second zebrafish Patched protein (Patched1) might compensate for the lack of a functional Patched2. While *patched1 (blowout)* mutant retinas contain all major retinal cell types, the lack of a retinal phenotype might be due to the holomorphic nature of the mutation (Koudijs, den Broeder

et al. 2008; Lee, Willer et al. 2008). However, this seems unlikely since *patched1* transcripts are not expressed at detectable levels in retinal tissue throughout development (*data not shown* and (Stenkamp, Frey et al. 2000)). The reduced number of differentiated Müller glia, a late born cell type (Schmitt and Dowling 1999), could feasibly be due to a bias towards early born cell types in *ptc2*$^{-/-}$ mutants; however, this potential bias does not affect neuronal composition of the retina at 5dpf. It is possible that a transient bias towards early neuronal cell types could occur early in retinal development, but any differences could masked by neurons added from the CMZ later in development. Conversely, any bias could also be present in neuronal populations that arise at the CMZ, resulting in an unnoticeable change in neuronal composition of the 5dpf *ptc2*$^{-/-}$ retina. With this in mind, it will be interesting to examine the proportion of retinal cell types in juvenile *ptc2*$^{-/-}$ mutants, as some homozygous fish reach two months of age (Koudijs, den Broeder et al. 2005). Finally, the Hh pathway could play a role in neuronal cell fate decisions in the retina through Hh ligand interactions with other Hh receptors. The Ihog Hh receptors have recently been suggested to compete with Patched for Hh binding (McLellan, Zheng et al. 2008) and act at levels at, or upstream of, Patched (McLellan, Yao et al. 2006). Indeed, the vertebrate Ihog homologue Cdo is expressed in the developing mouse retina (Mulieri, Okada et al. 2000). In the future, it would be interesting to test whether certain aspects of retinal development, such as cell fate decisions, might be Ihog-dependent.

II.3.2 *ptc2*$^{-/-}$ mutants as a possible model for the study of BCNS-related ocular pathologies

In BCNS patients, abnormalities at the interface between the retina and the vitreous, known as epiretinal membranes (ERMs), are associated with disruptions of the ILM and are thought to result from the overproliferation and ectopic presence of multiple cell types, including glia (Harada, Mitamura et al. 2006). BCNS ocular pathologies have been linked to mutations in the human *PTCH* gene (Hahn, Wicking et al. 1996; Johnson, Rothman et al. 1996; Black, Mazerolle et al. 2003), and have been shown to be associated with reactive Müller glia (Black, Mazerolle et al. 2003). Müller glial reactivity is often marked by upregulation of GFAP, and *ptc2*$^{-/-}$ mutants possessed regions of elevated GFAP levels. Elevated GFAP levels were consistently localized in the inner retina, where Müller glial endfeet terminate and contribute to the ILM. Staining with GS, which labels the Müller glial endfeet, revealed disruptions in ILM integrity in ~40% of mutant retinas assayed. In these cases, Müller glial endfeet did not terminate properly in the ILM, and the ILM was discontinuous. In some human patients, ERMs are found adjacent to retinal arteries (Black, Mazerolle et al. 2003); interestingly, we consistently detected the presence of reactive glial endfeet in localized retinal regions adjacent to the optic nerve and embryonic retinal vasculature. Importantly, no increases in cell death were detected in the retina or optic nerve of *ptc2*$^{-/-}$ mutants, or in Müller glia themselves (Figure II-6), ruling out reactivity due to apoptosis. Finally, in other contexts, Müller glia reactivity is often coupled with ectopic proliferation of these cells during 'reactive

gliosis' (Dyer and Cepko 2000). BrdU analysis did not detect ectopic proliferation in the central retinas of *ptc2*$^{-/-}$ mutants (Figure II-6 and Figure II-8), indicating that while a subset of these cells was reactive, they did not undergo a proliferative response. While reactive Müller glia have been shown to be associated with ERMs, it is unclear whether reactive Müller glia are the cause of these pathologies or, conversely, whether Müller glia become reactive in response to ERM formation. Further studies will be required to answer this question and to shed light on the cellular and molecular causes of ERM formation.

II.3.3 The retinal progenitor population of the CMZ is expanded in *ptc2*$^{-/-}$ mutants

Exact roles for Hh pathway activity in proliferating retinal progenitor cells remain unclear. Over-expression studies in *Xenopus* suggest a direct role for the Hh pathway in regulating proliferation by influencing the length of the cell cycle in CMZ progenitors (Locker, Agathocleous et al. 2006). In *Ptch1*$^{+/-}$ mice, more retinal progenitors are allocated to the CMZ throughout development and into adulthood (Moshiri and Reh 2004). BrdU pulse-chase analysis of the progenitor population at the *ptc2*$^{-/-}$ CMZ suggests that Patched-dependent Hh signaling controls the number of retinal progenitors in the zebrafish CMZ, and does not directly affect the length of the cell cycle in these cells. Importantly, cell counts at an earlier time-point, before the formation of the CMZ, revealed no increase in cell number in *ptc2*$^{-/-}$ retinas as compared to their wild-type siblings (Figure II-2E), suggesting that the increase in proliferating progenitors at the

CMZ is not simply due to an earlier proliferative event. Shh has been shown to control stem cell maintenance in multiple organs, including the adult brain (reviewed in (Fuccillo, Joyner et al. 2006)). Indeed, the Hh pathway genes *smo*, *gli2*, and *gli3* are expressed in the putative stem cell region of the *Xenopus* CMZ (Perron, Boy et al. 2003). In light of our findings, it is possible that the increase in retinal progenitors in the *ptc2*$^{-/-}$ CMZ is an indirect result of misregulation of the stem cell population rather than a direct effect on progenitor proliferation

CHAPTER III

Ectopic proliferation contributes to retinal dysplasia in the juvenile zebrafish *ptc2* mutant eye

III.1 INTRODUCTION

The Hedgehog (Hh) pathway is well known to control proliferation, differentiation, and patterning throughout the developing vertebrate central nervous system (Ekker, Ungar et al. 1995; Ericson, Muhr et al. 1995; Dahmane and Ruiz i Altaba 1999; Jiang and Hui 2008). In the vertebrate retina, Hh activity plays an integral role in the coordination of tissue growth and patterning through its influence on cell cycle progression of proliferating neuroblasts, which influences the timing of their cell cycle exit and differentiation (Ohnuma, Hopper et al. 2002; Locker, Agathocleous et al. 2006). Indeed, the mitogenic effects of the Hh pathway on retinal progenitors during development have been demonstrated in multiple model organisms (Wallace 2008). In zebrafish and *Xenopus* embryos, Hh pathway activation positively regulates cell cycle progression by influencing the lengths of the G1 and G2 phases of the cell cycle (Locker, Agathocleous et al. 2006). Consistent with these findings, Shh is required for the maintenance of retinal progenitor proliferation in mice(Wang, Dakubo et al. 2005; Sakagami, Gan et al. 2009).

The Hh receptor Patched is a tumor suppressor and negative regulator of the Hh pathway (Marigo, Davey et al. 1996; Stone, Hynes et al. 1996). Mutations in the human

PTCH gene have been linked to BCNS (OMIM 109400), a disorder characterized by dental, skeletal, and radiographic defects, as well as a predisposition to early- and late-onset tumorigenesis (reviewed in (High and Zedan 2005)). Early lethality of *Ptch1*$^{-/-}$ mice (Goodrich, Milenkovic et al. 1997), an established model for BCNS (Goodrich, Milenkovic et al. 1997; Black, Mazerolle et al. 2003), has limited the investigation of the effects of loss of Patched function to largely developmental contexts. Interestingly, post-natal *Ptch1*$^{+/-}$ mice possess retinal dysplasias and epiretinal membranes, structural abnormalities arising at the vitreo-retinal interface. Dysplasic regions of the retina in *Ptch1*$^{+/-}$ mice are characterized by foci of disrupted lamination and the presence of photoreceptor 'rosettes' in the INL (Black, Mazerolle et al. 2003). Ectopic proliferation of retinal cells is thought to account for retinal dysplasias in *Ptch1*$^{+/-}$ mice; however, this relationship has not yet been experimentally demonstrated.

In addition to retinal dysplasia and vitreo-retinal abnormalities, post-natal *Ptch1*$^{+/-}$ mice also possess increased proliferation in the retinal margin (Moshiri and Reh 2004). In lower vertebrates, such as fish, frogs and chicks, a population of proliferative cells at the retinal margin called the CMZ continues to contribute to the growth of the retina throughout the life of the animal (Negishi, Teranishi et al. 1982; Wetts, Serbedzija et al. 1989). The spatial pattern of cells within the CMZ- retinal stem cells proximally, followed by proliferative retinal progenitors centrally and differentiating progenitors more distally, is thought to recapitulate the temporal sequence of early retinal development (Wetts, Serbedzija et al. 1989; Perron, Kanekar et al. 1998). Loss of a

single allele of the mouse *Ptch1* gene curiously results in the persistence of a population of retinal progenitors very reminiscent of the CMZ, a structure normally absent in mammals (Moshiri and Reh 2004).

The zebrafish *ptc2*^{tj222} (the orthologue of the mammalian *Ptch1* (Lewis, Concordet et al. 1999)) mutant was isolated in a genetic screen designed to identify genes involved in the regulation of proliferation during embryonic development (Koudijs, den Broeder et al. 2005). *ptc2*^{tj222} mutants possess a nonsense mutation in the exon encoding the sixth trans-membrane domain of Patched2, which is predicted to abolish its function (Johnson, Milenkovic et al. 2000; Koudijs, den Broeder et al. 2005). Loss of Patched function results in an overactive Hh pathway due to derepression of Smoothened, a transmembrane protein responsible for transducing Hh signaling (van den Heuvel and Ingham 1996; Murone, Rosenthal et al. 1999).

Our previous characterization of embryonic retinal development in *ptc2* mutants revealed vitreo-retinal abnormalities, similar to those observed in *Ptch1*$^{+/-}$ mice, and in human BCNS patients. Our findings suggested that the vitreo-retinal abnormalities were developmental in origin (Bibliowicz and Gross 2009). Other aspects of retinal development such as retinal lamination were normal up to late embryonic stages (5 days post-fertilization, 5dpf); however, an increase in the number of proliferative progenitors at the CMZ was observed, suggesting a role during embryonic eye development for

Patched2 in negatively regulating the size of this retinal progenitor/stem cell population (Bibliowicz and Gross 2009).

A small percentage of zebrafish *ptc2*$^{-/-}$ mutants survive to juvenile stages (Koudijs, den Broeder et al. 2005). Given that retinal dysplasias do not appear in *Ptch1*$^{+/-}$ mice until post-natal stages and the cellular underpinnings of this phenotype is not known, we utilized juvenile *ptc2*$^{-/-}$ mutants to gain further insight into the consequences of loss of Patched function on the growth and patterning of the post-embryonic retina. Our analyses identified peripheral retinal dysplasias in juvenile *ptc2*$^{-/-}$ mutants that included disrupted lamination, the presence of ectopic clusters of cells in the INL, and morphological abnormalities at the retinal margin. Analysis of cell proliferation in the *ptc2*$^{-/-}$ juvenile retina revealed overproliferation in all retinal regions where proliferative progenitors normally reside, i.e. the CMZ, INL, and outer nuclear layer (ONL). Continually proliferative progenitors were detected in *ptc2*$^{-/-}$ mutants adjacent to the CMZ, and proliferative cells were associated locally with dysplasic retinal regions that contained ectopic neurons. Importantly, BrdU pulse-chase assays, combined with immunohistochemistry for retinal neuronal markers, demonstrated that ectopic retinal neurons within these dysplasic regions originated from an ectopic proliferative event. Taken together, our findings support a role for Patched2 in negatively regulating proliferation in the post-embryonic retina and demonstrate that ectopic proliferation in the *ptc2*$^{-/-}$ juvenile retina directly contributes to retinal dysplasias in these mutants.

III.2 RESULTS

III.2.1 Juvenile *ptc2*$^{-/-}$ mutants possess peripheral retinal dysplasias and abnormalities at the CMZ and ciliary zone

To begin to investigate the effect of the *ptc2* mutation on the post-embryonic zebrafish retina, histological analyses were performed on *ptc2*$^{-/-}$ mutants and their heterozygous and wild-type siblings. While most *ptc2*$^{-/-}$ fish do not reach adulthood, ~2% survive to 6 weeks of age ("juveniles"). Juvenile *ptc2*$^{-/-}$ mutants were smaller than their wild-type siblings and had mild pigmentation defects, overall eye structure appeared normal (Figure III-1A). Juvenile *ptc2*$^{+/-}$ fish were indistinguishable from their wild-type siblings (data not shown) and when examined histologically, retinal morphology and lamination also appeared to be normal (n=6, Figure III-1C), when compared to wild-type siblings (Figure III-1B). In *ptc2*$^{-/-}$ mutants, however, regions of disrupted retinal lamination were detected in the dorsal peripheral retina, as well as morphological abnormalities in the CMZ and ciliary zone (Figure III-1D). Of the ten *ptc2*$^{-/-}$ juveniles that were analyzed by histology, four contained ectopic clusters of cells that appeared to be continuous with the CMZ (n=4/10, Figure III-1E,F). These ectopic cells were incorrectly incorporated into the retina and disrupted normal retinal lamination. Three other *ptc2*$^{-/-}$ juveniles contained clusters of ectopic cells within the INL; however,

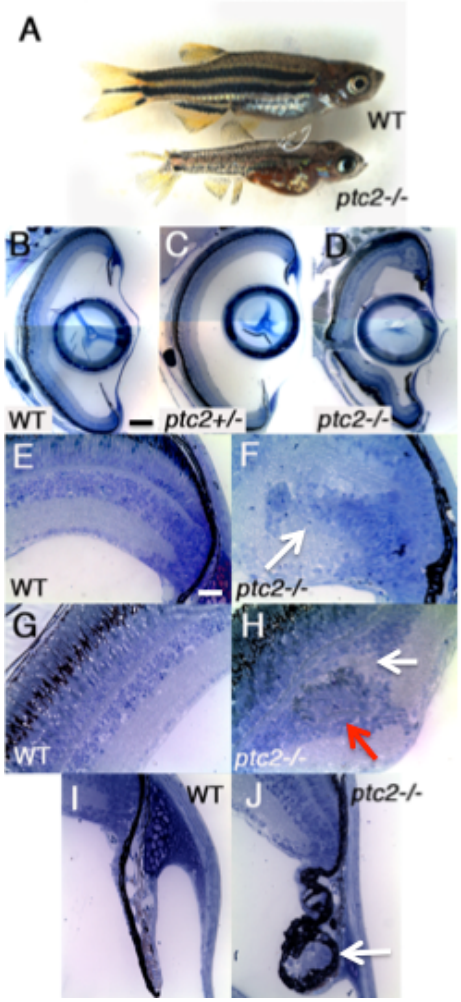

Figure III-1. Juvenile *ptc2*$^{-/-}$ mutants possess peripheral retinal dysplasias and abnormalities at the ciliary zone. A small percentage of *ptc2*$^{-/-}$ mutants (~2%) reach 6 weeks of age. *ptc2*$^{-/-}$ mutants are smaller than their wild-type siblings and have disrupted pigmentation pattern (A). Histological analysis of wild-type (B,E,G,I), *ptc2*$^{+/-}$ (C), and *ptc2*$^{-/-}$ (D,F,H,J) 6 week retinas. Retinal organization appears normal in *ptc2*$^{+/-}$ mutants (C). In *ptc2*$^{-/-}$ mutants, retinal disorganization was found in the dorsal peripheral retina, while the rest of the retina was laminated normally (D). Within the dorsal peripheral retina of *ptc2*$^{-/-}$ mutants, ectopic neurons that appeared to be continuous with the CMZ and disrupted lamination were detected (arrow in F, n=4/10). Ectopic neuronal clusters in the INL were also observed (H, n=3/10). Neuronal clusters contained photoreceptor outer segments (red arrow in H) and were associated with an ectopic plexiform layer (white arrow in H). Morphological abnormalities and ectopic cells (arrow in J) were observed in the ciliary zone (n=3/10). Scale bars 100um (B-D) and 25um (E-J).

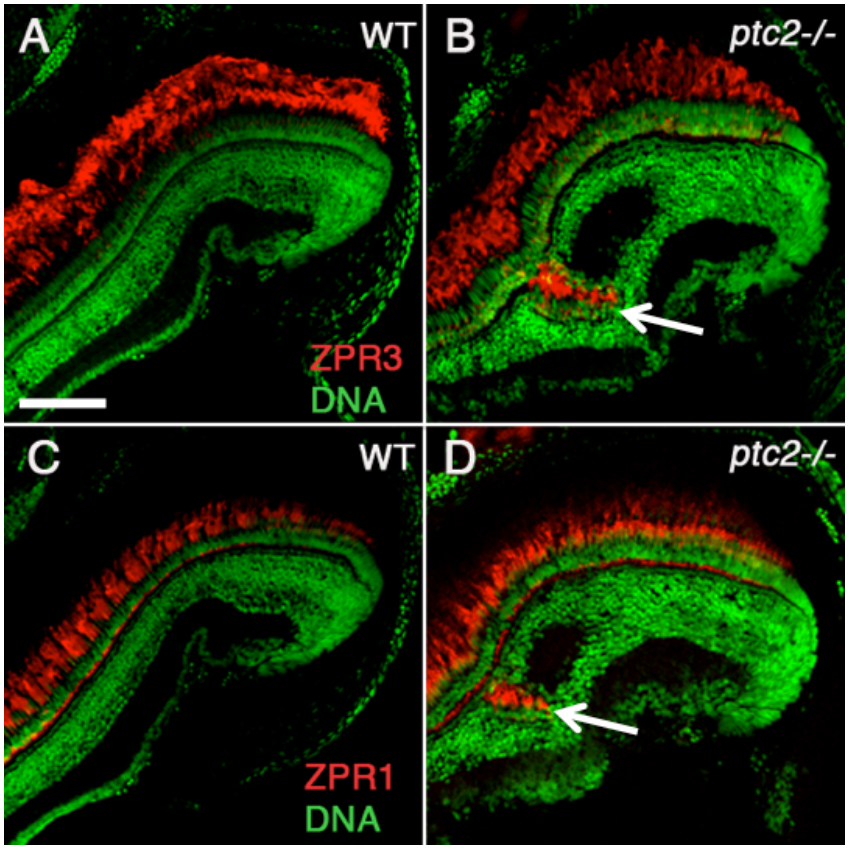

Figure III-2. Ectopic neuronal clusters in juvenile *ptc2*$^{-/-}$ retinas contain rod and cone photoreceptors. Both ZPR3-expressing rods (arrow in B) and ZPR1-expressing red/green double cones (arrow in D) are detected in the ectopic neuronal clusters in *ptc2*$^{-/-}$ retinas, while in wild-type retinas (A,C), photoreceptors are restricted to the ONL. Scale bar 100um.

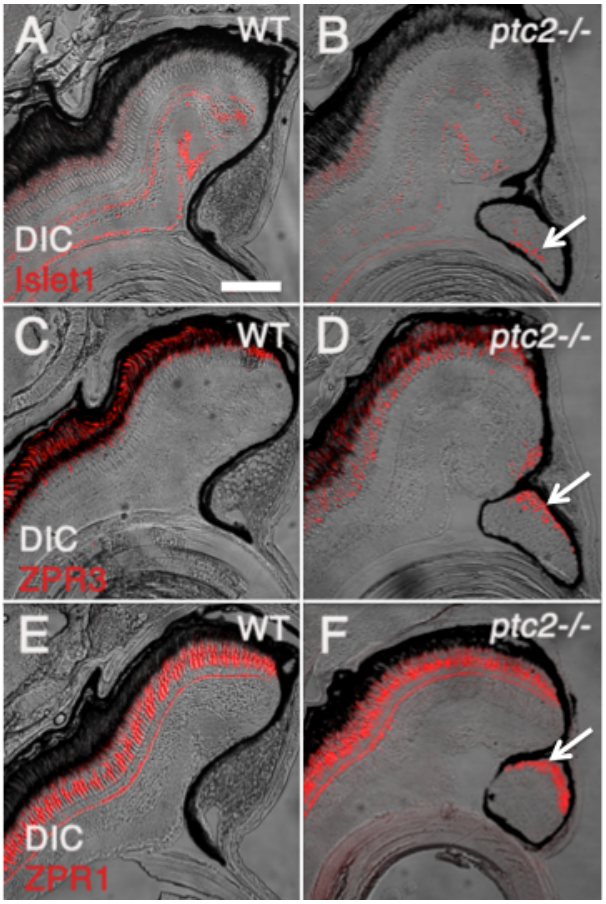

Figure III-3. Ectopic cells at the *ptc2*$^{-/-}$ juvenile ciliary zone label with markers of differentiated retinal neurons. Immunohistochemical analysis of *ptc2*$^{-/-}$ juvenile retinas revealed cells at the ciliary zone that are positive for the neuronal markers Islet1 (B), ZPR3 (rods, D) and ZPR1 (red/green double cones, F),. In wild-type sibling retinas, staining was restricted to the retina (A,C,E). Scale bar: 100um.

lamination in the remainder of the retina in these individuals appeared to be normal (n=3/10, Figure III-1G,H). Examination of sequential histological sections through the depth of the retina indicated that these clusters did not appear to be continuous with the CMZ (data not shown). These ectopic clusters contained photoreceptors, indicated by the presence of photoreceptor outer segments, and they were associated with an ectopic plexiform layer (arrows in Figure III-1H). Immunohistochemical analyses confirmed the presence of both cone and rod photoreceptors within these dysplasic foci (Figure III-2). Additionally, five of the ten *ptc2*$^{-/-}$ juveniles examined possessed abnormalities in the 'ciliary zone', a region located at the base of the iris which contains a non-pigmented epithelium continuous with the CMZ (n=5/10) (Soules and Link 2005). These abnormalities varied in severity between affected individuals and were characterized by the presence of ectopic cells surrounded by pigmented tissue (Figure III-1I,J). Immunohistochemical analyses revealed that this abnormal ciliary zone contained differentiated neurons characteristic of the neural retina (Figure III-3).

III.2.2 The juvenile *ptc2* retina is overproliferative

The Hh pathway promotes retinal progenitor proliferation in multiple model organisms (Jensen and Wallace 1997; Moshiri and Reh 2004; Moshiri, McGuire et al. 2005), and in *Ptch1*$^{+/-}$ mice, ectopic proliferation has been observed both within the mature retina, where proliferation is normally minimal at post-embryonic stages, and in the retinal periphery (Black, Mazerolle et al. 2003; Moshiri and Reh 2004). Indeed,

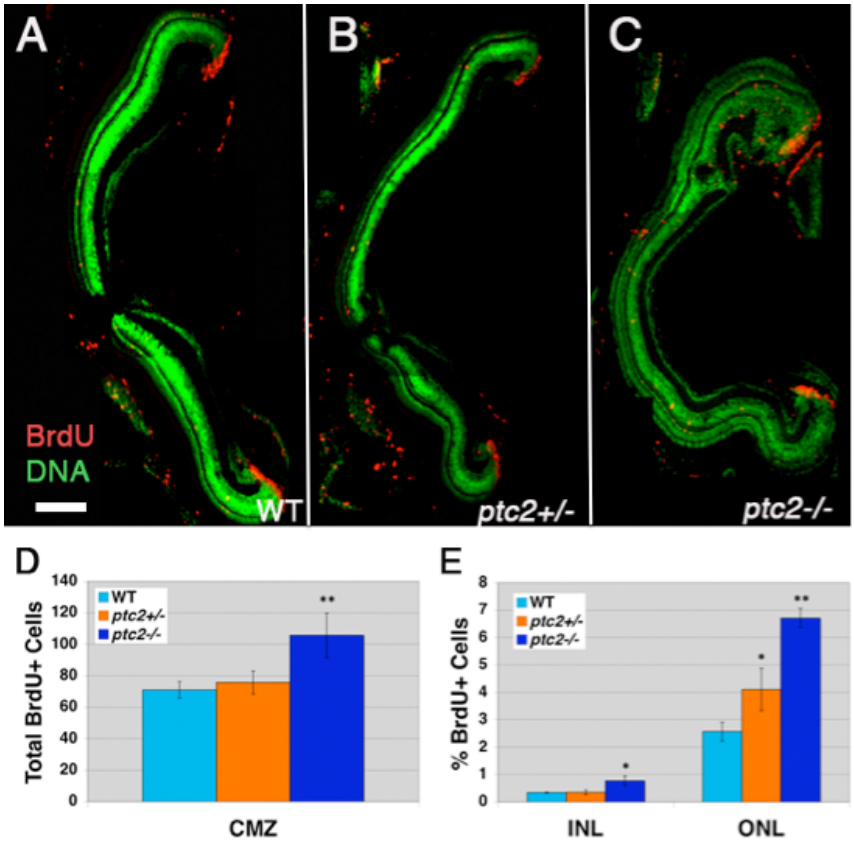

Figure III-4. The *ptc2*$^{-/-}$ retina is overproliferative. BrdU incorporation (2 hours) at 6 weeks revealed an increase in the number of proliferative cells in the *ptc2*$^{-/-}$ retina (C) when compared to wild-type (A) (n=4). The total number of BrdU+ cells in the CMZ was increased by 54% in *ptc2*$^{-/-}$ mutants when compared to wild-type siblings (**p<0.001, n=4)(D). The percentage of BrdU+ cells in the INL (2.30-fold, *p<0.01, n=4) and ONL (2.62-fold, **p<0.001, n=4) were significantly increased in *ptc2*$^{-/-}$ mutants when compared to wild-type siblings (E). A statistically significant increase in the percentage of BrdU+ cells was also observed in the ONL of *ptc2*$^{+/-}$ mutants (B,E)(1.54-fold, *p<0.01, n=4) when compared to wild-type (A,E). Scale bar 100um.

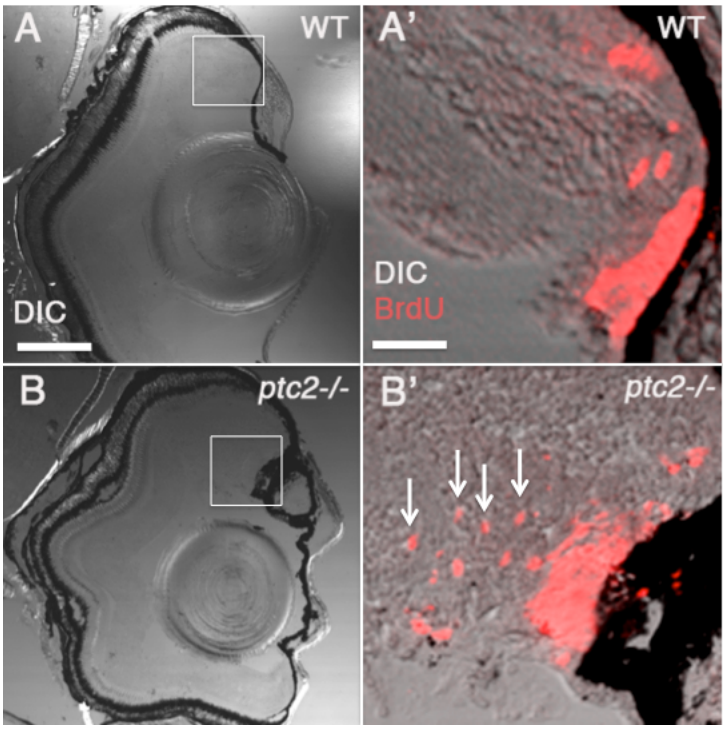

Figure III-5. Ectopic proliferation at the *ptc2*$^{-/-}$ juvenile retinal periphery. BrdU incorporation (2 hours) at 6 weeks revealed proliferative cells within the peripheral *ptc2*$^{-/-}$ retina, adjacent to the CMZ (B, arrows in high magnification of boxed area in B'). In wild-type retinas, proliferative cells at the retinal periphery are confined to the CMZ and newly generated rods (A, high magnification in A'). Scale bars 100um (A,B) and 40um (A',B').

ectopic proliferation is thought to underlie the formation of retinal dysplasia in *Ptch1*$^{+/-}$ mice (Black, Mazerolle et al. 2003); however, a direct relationship between proliferation and retinal dysplasia has not been experimentally established. To begin to address this question, it was first necessary to determine if proliferation was indeed perturbed in juvenile zebrafish *ptc2*$^{-/-}$ mutants.

To quantify proliferation, a BrdU incorporation assay was performed on *ptc2*$^{-/-}$, *ptc2*$^{+/-}$ and wild-type siblings. 6-week old fish were exposed to BrdU for 2 hours, sacrificed and the percentage of BrdU+ cells out of total cells was determined for the INL and outer nuclear layer (ONL), while the total number of BrdU+ cells was determined for the CMZ. Statistically significant increases in the percent of BrdU+ cells were observed in *ptc2*$^{-/-}$ mutants in the INL (n=4, 2.30-fold increase, $p<0.01$) and ONL (n=4, 2.62-fold increase, $p<0.001$) (Figure III-4A,C,E), when compared to wild-type fish. In addition, the total number of BrdU+ cells in the CMZ was increased by 54% in *ptc2*$^{-/-}$ mutants when compared to wild-type siblings (n=4, Figure III-4A,C,D). Interestingly, overproliferation within the INL was mostly confined to the retinal periphery (Figure III-5), where proliferation is normally restricted to a small population of Müller glia. In the INL, Müller glia proliferation is thought to give rise to rod progenitor cells that then migrate to the ONL and differentiate into rod photoreceptors (Otteson, D'Costa et al. 2001; Bernardos, Barthel et al. 2007). Overproliferation in the INL could therefore be due to Müller glia overproliferation, while the increase in BrdU+ cells in the ONL suggests an overproduction of rod progenitors. Finally, while no differences in the INL

or CMZ were observed in *ptc2*$^{+/-}$ fish, a 1.58-fold increase in the percentage of BrdU+ cells in the ONL was observed when compared to siblings (n=4, p<0.01, Figure III-4B,D,E).

III.2.3 *ptc2* is expressed in the progenitor/stem cell populations of the juvenile retina

Given the overproliferation phenotypes in *ptc2*$^{-/-}$ mutants, we next wanted to define the cell types in which *ptc2* was expressed in the juvenile eye. *In situ* hybridization for *ptc2* transcripts was performed on retinal cryosections from 6-week old wild-type embryos. *ptc2* expression was observed in a discrete population of cells located within the INL (Figure III-6B,D). In mouse, *Ptch1* (the orthologue of the zebrafish *ptc2* (Lewis, Concordet et al. 1999)) is expressed in Müller glia (Wang, Dakubo et al. 2002), and the location of *ptc2*-expressing cells in the zebrafish INL suggested that they might be Müller glia. To determine if zebrafish Müller glia express *ptc2*, a *gfap::GFP* transgenic line was utilized that expresses GFP in Müller glia (Bernardos and Raymond 2006; Qin, Barthel et al. 2009). GFP+ and GFP- cells were isolated from dissociated retinas by FACS, and quantification of *ptc2* levels by qRT-PCR in GFP+ and GFP- cell populations revealed a 3.72-fold enrichment in GFP+ cells (n=3, p<0.01, Figure III-6E).

We previously reported *ptc2* expression in the most peripheral region of the CMZ in embryonic zebrafish (Bibliowicz and Gross 2009), and in *Ptch1lacZ*+/- mice, *lacZ*

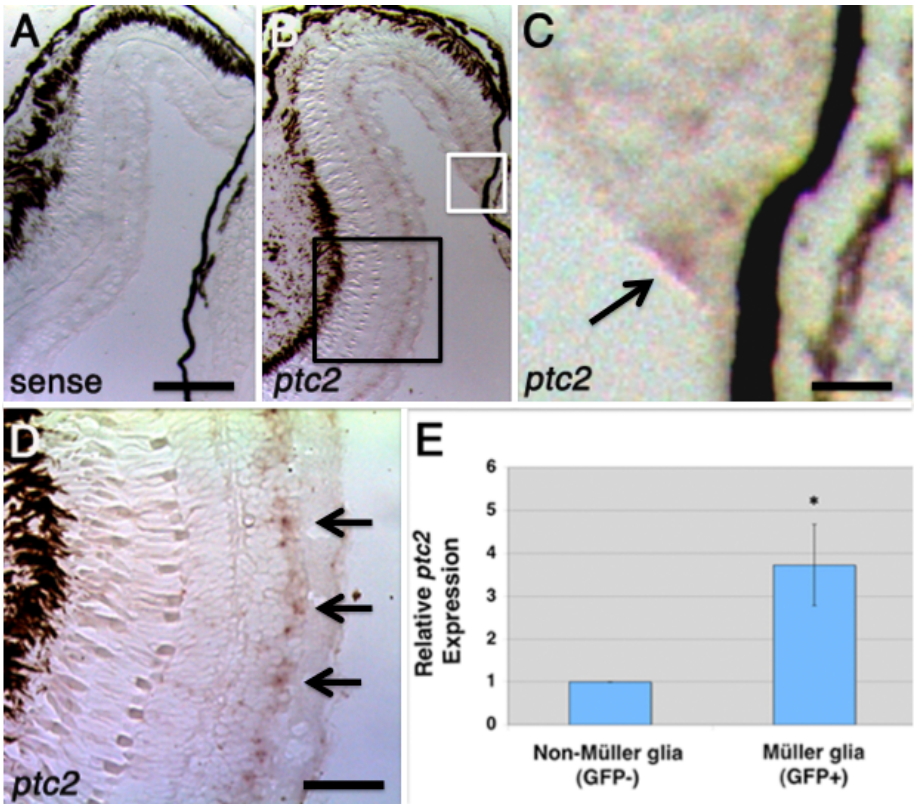

Figure III-6. *ptc2* is expressed in progenitor/stem cell populations of the juvenile zebrafish retina. *In situ* hybridization for the *ptc2* transcript in 6 week old juvenile wild-type retinas. Staining was absent throughout the retina when hybridization was performed using a *ptc2* sense control probe (A). *ptc2* expression was detected in the retinal margin (B, high magnification of white box in C), where CMZ progenitors reside, and in a population of cells in the INL (B, and high magnification of the black box in D). qRT-PCR analysis of *ptc2* expression from FACS sorted GFP+ Müller glia cells isolated from a *gfap:GFP* transgenic line revealed enrichment of the *ptc2* transcript in GFP+ Müller glia when compared to GFP-, non-Müller glial cells (E, *$p<0.01$, n=3). Scale bars: 100um (A,B), 25um (C) and 50um (D).

staining is detected in a small population of cells at the retinal margin (Moshiri and Reh 2004). In juvenile zebrafish, *ptc2* was also detected at low levels in a few cells at the CMZ (Figure III-6B,C). Thus, *ptc2* is expressed in the two progenitor/stem cell populations of the post-embryonic zebrafish retina, the CMZ and Müller glia, suggesting that it could have a role within these cell populations to regulate proliferation.

III.2.4 Peripheral dysplasias in the *ptc2*$^{-/-}$ juvenile retina contain continually proliferative cells that do not express Müller glia markers

BrdU incorporation assays revealed proliferative cells in the peripheral retina, adjacent to the CMZ (Figures III-4, III-5), and therefore, overproliferation at the *ptc2*$^{-/-}$ retinal periphery might be due to CMZ-derived proliferative progenitors that abnormally remain in the cell cycle. Alternatively, Müller glia, which function as endogenous progenitors in the post-embryonic retina (Bernardos, Barthel et al. 2007), could be overproliferative. Indeed, in zebrafish, immature Müller glia express higher levels of stem cell markers and are more proliferative than their mature, centrally located, counterparts (Julian, Ennis et al. 1998; Raymond, Barthel et al. 2006). Moreover, Shh promotes the proliferation of rat Müller glia *in vitro* (Wan, Zheng et al. 2007), suggesting that Müller glia proliferation might be sensitive to Hh pathway activity.

To address these possibilities, a *ptc2;gfap::GFP* line was generated and a BrdU/EdU 'pulse-chase-pulse' experiment was performed in this line (Figure III-7A).

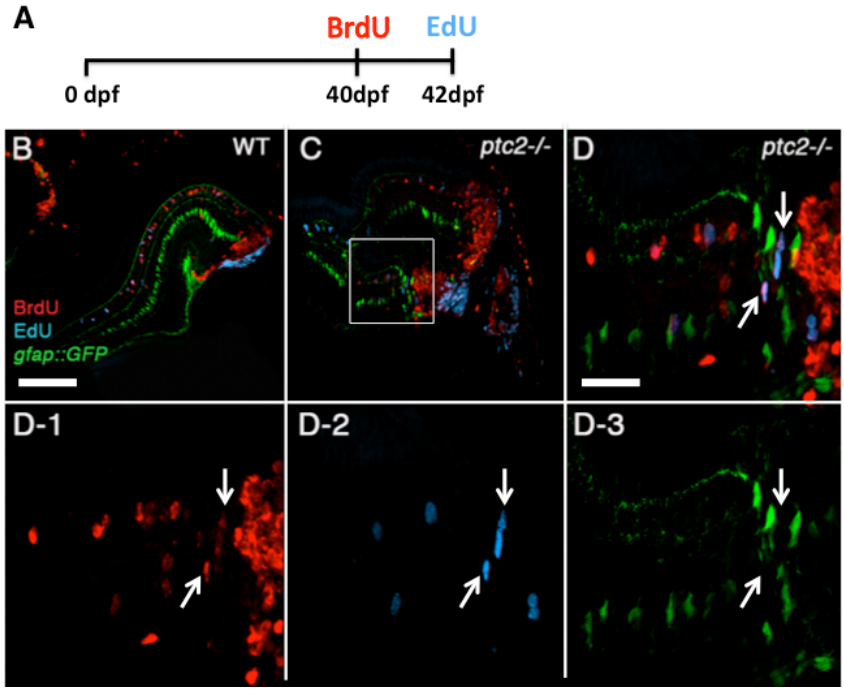

Figure III-7. Continually proliferative cells in regions of retinal dysplasia in *ptc2*$^{-/-}$ mutants. A BrdU/EdU 'pulse-chase-pulse' experiment was performed on wild-type *gfap:GFP* (B) and *ptc2*$^{-/-}$*;gfap:GFP* mutant retinas (C,D,D1-3). Fish were exposed to BrdU for 8 hours at 40 days post fertilization (dpf), returned to their tanks for two days, injected with EdU at 42dpf (6 weeks), and fixed for BrdU and EdU immunohistochemistry and detection (A). In wild-type retinas, cells that were proliferative at time of fixation (EdU+) were mostly confined to the CMZ, while cells that were proliferative two days before fixation (BrdU+) have incorporated into the retina. In addition, a few GFP+ Müller glia were also either BrdU+ or EdU+ (B). In *ptc2*$^{-/-}$*;gfap:GFP* retinas, in addition to BrdU+/GFP+ or EdU+/GFP+ Müller glia, BrdU+/EdU+ double-labeled cells were detected in the peripheral retina, and these did not express GFP (C, white arrows in high magnification of boxed region in D and in D1-D3, n=3/3). Scale bars 100um (B,C) and 20um (D,D1-D3).

Briefly, 6-week old fish were 'pulsed' with BrdU for eight hours, returned to their tanks for two days (chase) and then injected with EdU (pulse). After a four-hour recovery, the fish were euthanized, fixed and processed for immunohistochemistry. In this assay, continually proliferative cells would be BrdU+/EdU+, while those that ceased proliferation after the first exposure would only be BrdU+. The presence of GFP would suggest a Müller glial identity, while an absence will suggest a non-Müller glial origin. In the wild-type retina, BrdU+ cells were properly incorporated into the differentiated retina and a few BrdU+/GFP+ or EdU+/GFP+ cells were observed, indicating proliferative Müller glia (Figure III-7B, and data not shown). EdU+ cells were largely restricted to the CMZ and no BrdU/EdU+ cells were detected outside of the CMZ. Of the ten *ptc2*$^{-/-}$*;gfap::GFP* fish analyzed, three possessed retinal dysplasia. In all three of these fish, beyond the normal populations of BrdU+ or EdU+ cells, several BrdU+/EdU+ cells were detected in the retinal periphery, physically separated from the EdU+ cells of the CMZ (n=3/3, Figure III-7C,D,D1-D3). Importantly, these BrdU+/EdU+ cells did not co-localize with GFP, indicating that they were not likely to be Müller glia (Figure III-7D,D1-D3).

III.2.5 Ectopic neuronal clusters in regions of retinal dysplasia are associated with ectopic proliferation

We next wanted to investigate the relationship between proliferation and the formation of ectopic photoreceptor-containing neuronal clusters observed in the *ptc2*$^{-/-}$

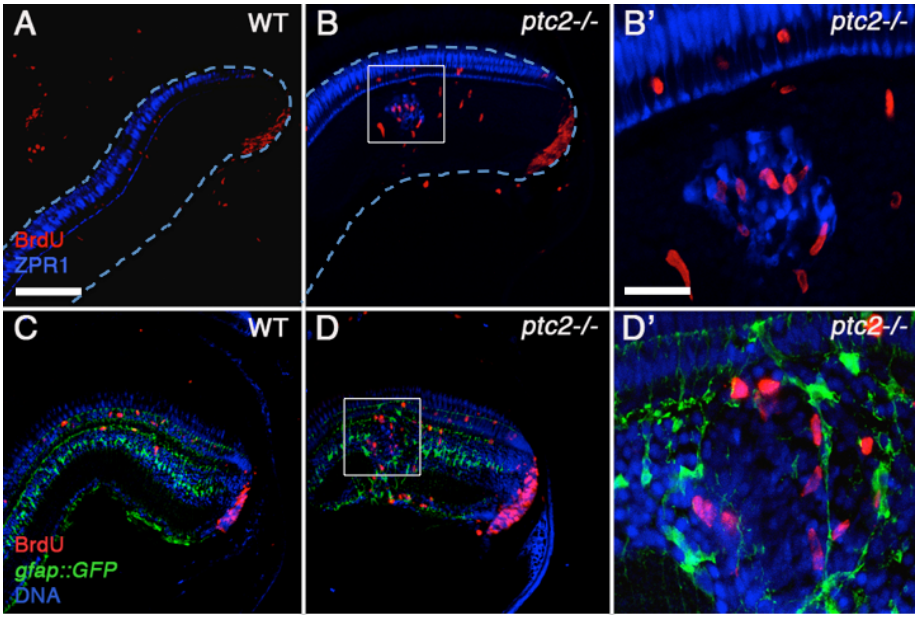

Figure III-8. Ectopic neuronal clusters in regions of retinal dysplasia are locally associated with ectopic proliferation. Immunohistochemical analysis of wild-type retinas after a two-hour exposure to BrdU revealed few proliferative cells in the INL (A,C), while in dysplasic regions of the *ptc2*$^{-/-}$ retina, BrdU+ cells were locally associated with ectopic ZPR1-expressing red/green double cones in the INL (B, high magnification of white box in B'). Analysis of adjacent sections from the same individuals showed that these proliferative cells were not Müller glia, as they did not label with GFP in the *gfap:GFP* transgenic background (D, high magnification of white box in D'). Scale bar: 100um.

retina. Examination of multiple neuronal clusters in the INL revealed ectopic proliferation that was locally associated with these cells (n=4/5) (Figure III-8A,B,B'). In the *Ptch1*$^{+/-}$ mouse retina, rosettes are associated with Müller glial reactivity that is thought to occur in response to the presence of the rosettes (Soules and Link 2005). Reactivity is often characterized by the up-regulation of GFAP, and sometimes by increased proliferation (Sahel, Albert et al. 1990). In order to determine whether proliferation around the neuronal clusters in *ptc2*$^{-/-}$ juveniles resulted from Müller glial reactivity, we examined the spatial relationship between proliferative cells, Müller glia and regions of retinal dysplasia within the INL of *ptc2*$^{-/-}$*;gfap::GFP* juveniles (Figure III-8C,D,D'). While some BrdU+ cells were located adjacent to GFP+ Müller glia, co-localization of BrdU and GFP was not observed in any of these clusters, suggesting that proliferating cells associated with the ectopic neuronal clusters were not Müller glia (n=0/5) (Figure III-8D,D'). This finding was also corroborated by the lack of colocalization for GFAP and BrdU using an anti-GFAP antibody in immunohistochemical assays (data not shown).

III.2.6 Ectopically proliferating cells generate new neurons in the dysplasic regions of the *ptc2*$^{-/-}$ retina

Results thus far indicate that ectopic proliferation is locally associated, and correlates, with dysplasic regions of the *ptc2*$^{-/-}$ retina. However, whether these ectopically proliferative cells actually generate the ectopic neurons and thereby contribute to the

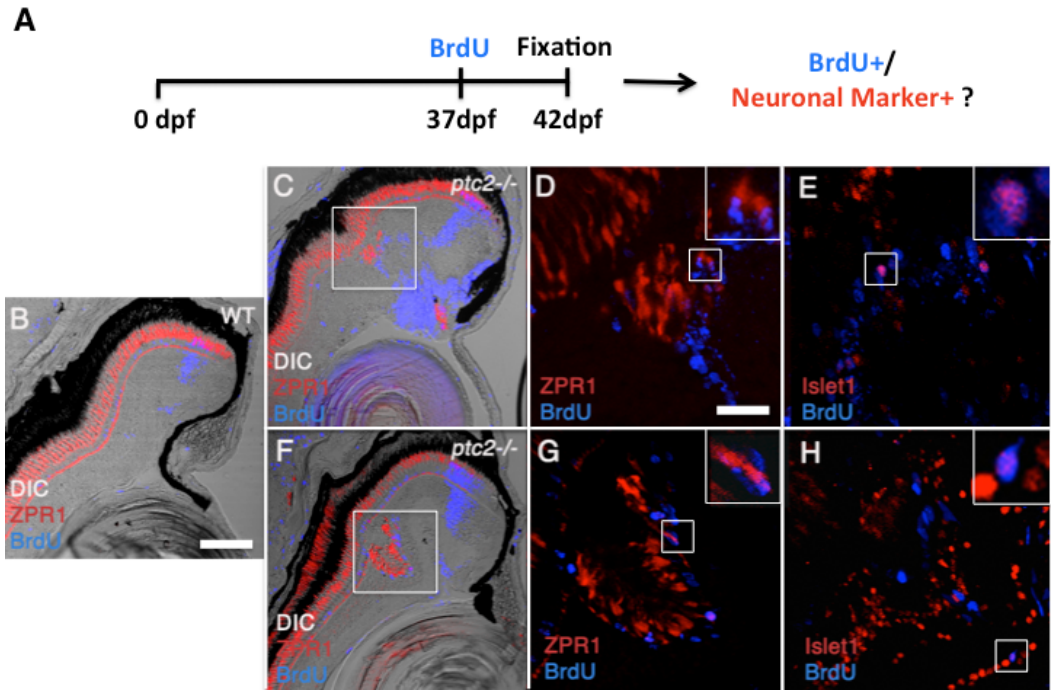

Figure III-9. New neurons are generated by ectopic proliferation in the dysplasic *ptc2*$^{-/-}$ juvenile retina. A combined BrdU pulse-chase and immunohistochemical assay to determine if ectopically proliferative cells generate new neurons in dysplasic regions of the *ptc2*$^{-/-}$ retina (A). Fish were exposed to BrdU for eight hours at 37dpf, returned to their tanks for five days, and fixed for immunohistochemistry at 42dpf (6 weeks). In wild-type siblings (B), BrdU+ cells were detected in a narrow band within the differentiated retina, corresponding to CMZ-derived cells that were proliferative five days before fixation, as well as in scattered rod progenitors located in the ONL. Red/green double cones, marked by ZPR1, are restricted to the ONL. In two different *ptc2*$^{-/-}$ retinas, BrdU+ cells colocalize with ZPR1 (C,F, high magnification of boxed areas in D,G). In adjacent sections from the same individuals, BrdU+ cells are also positive for Islet1, a marker of retinal ganglion cells, and subsets of differentiated amacrine, bipolar and horizontal cells (E,H). Insets in D,E,G and H are higher magnification of the respective boxed regions showing co-localization of BrdU and the neuronal marker. Scale bars 100um (B,C,F) and 20um (C,E,G,H).

formation of retinal dysplasias in *ptc2*$^{-/-}$ mutants is still not known. To directly analyze this possibility, we performed a BrdU pulse-chase assay combined with immunohistochemistry for the detection of differentiated retinal neurons, to determine if proliferative cells differentiated into new neurons in *ptc2*$^{-/-}$ retinas. Experimentally, *ptc2*$^{-/-}$ juveniles, and their wild-type siblings, were exposed to BrdU for 8 hours at the age of 37 days, returned to their tanks for five addition days, and fixed for immunohistochemical analysis at 42 days of age (Figure III-9A). In wild-type siblings, a population of BrdU+ cells was evident as a tight band of cells within the differentiated retina. These BrdU+ cells corresponded to CMZ-derived progenitors that were proliferative at day 37, but by 42dpf had differentiated and become incorporated into the retina (Figure III-9B). Analysis of *ptc2*$^{-/-}$ juveniles in this assay revealed dysplasias in six of the twenty-two individuals analyzed. In five of the six *ptc2*$^{-/--}$ mutants with dysplasias, zpr1-positive red/green double cone photoreceptor outer segments were directly associated with BrdU-positive nuclei (n=5/6, Figure III-9C,D,F,G). Unlike rod photoreceptors that are produced from rod progenitors within the differentiated retina, cone photoreceptors are only produced in the CMZ. Thus, our findings indicate that ectopically proliferative cells can generate cone photoreceptors in juvenile *ptc2*$^{-/-}$ mutants.

To further determine if neurons within dysplasic regions arise from ectopically proliferating cells, adjacent sections to those used for zpr1 immunohistochemistry were stained for Islet1, a marker of retinal ganglion cells, and subsets of differentiated amacrine, bipolar and horizontal cells (Link, Fadool et al. 2000; Shkumatava, Fischer et

al. 2004). Indeed, in these sections, Islet1 co-localized with BrdU (n=3/4, Figure III-9E,H), indicating that ectopically proliferating cells in the *ptc2*$^{-/-}$ mutant retina also generated Islet1-expressing neurons. Taken together, these results point to ectopic proliferation as the source of ectopic neurons in the *ptc2*$^{-/-}$ retina, and as the likely cellular underpinning of the retinal dysplasias observed in juvenile *ptc2*$^{-/-}$ mutants.

III.3 DISCUSSION

III.3.1 Ectopic proliferation contributes to retinal dysplasia in the juvenile *ptc2* retina

Our analyses have revealed abnormalities in the juvenile *ptc2*$^{-/-}$ mutant zebrafish retina, confined to the peripheral retina, that included ectopic proliferation and retinal dysplasias, composed of ectopic neuronal clusters, within the INL. These phenotypes are similar to those observed in the dysplasic *Ptch1*$^{+/-}$mouse retina (Black, Mazerolle et al. 2003; Moshiri and Reh 2004). From studies of the *Ptch1*$^{+/-}$ mouse it has been hypothesized that retinal dysplasias could result from retinal progenitor overproliferation. Our results demonstrate that retinal neurons within the dysplasic regions of the *ptc2*$^{-/-}$ juvenile retina do indeed arise from an ectopic proliferative event, supporting a model in which loss of Patched 2 function leads to overproliferation of cells within the retina, ultimately contributing to retinal dysplasias in *ptc2*$^{-/-}$ mutants.

It is of interest, considering the known role of Patched as a tumor suppressor (Hahn, Wicking et al. 1996; Johnson, Rothman et al. 1996), that retinal phenotypes in the *ptc2* juvenile retina occur in regions that are either known to, or thought to, contain retinal stem cells. While the lineage of the ectopically proliferating cells in the *ptc2*$^{-/-}$ juvenile retina is still unknown, expression of *ptc2* in both the CMZ and in Müller glia suggests that one of these progenitor/stem cell populations might be the source of these proliferative cells. The presence of ectopically proliferating cells adjacent to the CMZ (Figure III-7), as well as locally associated with neuronal clusters (Figure III-8) in the *ptc2*$^{-/-}$ juvenile retina, may account for the increased number of proliferative cells in the INL. Unlike the CMZ which gives rise to multiple retinal cell types, Müller glia give rise to lineage restricted rod progenitors that then migrate to the ONL and differentiate into rod photoreceptors (Otteson, D'Costa et al. 2001; Bernardos, Barthel et al. 2007). However, upon exposure to growth factors or in response to physical injury to the retina, Müller glia are thought to de-differentiate, proliferate, and give rise to all neuronal cells types (Fischer and Reh 2003; Das, Mallya et al. 2006; Bernardos, Barthel et al. 2007). Analysis of proliferation and retinal dysplasia in *ptc2*$^{-/-}$*;gfap::GFP* transgenic fish revealed that ectopically proliferating cells were not likely to have been derived from Müller glia. Moreover, preliminary lineage tracing studies utilizing *ptc2*$^{-/-}$ *;tuba1a*::*CreER*^{T2}*; β-actin2::LCLG* transgenic fish (Ramachandran, Reifler et al. 2010) have not detected Müller glia derived cells in *ptc2*$^{-/-}$ mutants (JB, unpublished observations). While we cannot exclude the possibility that the ectopically proliferating cells in juvenile *ptc2*$^{-/-}$ mutant retinas are of Müller glia origin, our findings to date do not

support this possibility. Identification of the cellular origin of the these ectopic neurons can only be unambiguously completed using targeted transgenic lineage tracing tools, and multiple cellular origins for the ectopically proliferating cells are possible; however, our results suggest that misregulation of progenitor cell proliferation in the CMZ is very likely to contribute to the process, and lead to the formation of ectopic neurons and retinal dysplasias in *ptc2*$^{-/-}$ mutants.

While the mitogenic role of Patched during embryonic neural development is well established (Hahn, Christiansen et al. 1996; Goodrich, Milenkovic et al. 1997), less is known regarding its function in the post-embryonic central nervous system (CNS). In the post-natal mouse cerebellum, the Hh pathway promotes precursor cell proliferation(Wallace 1999; Wechsler-Reya and Scott 1999), and medulloblastomas, tumors of the cerebellum, have been described in both *Ptch1*$^{+/-}$ mice and in human BCNS patients(Peringa, Fung et al. 1995; Goodrich, Milenkovic et al. 1997) . In mouse, loss of Ptch1 function results in increased retinal proliferation, suggesting that Ptch1 acts to negatively regulate progenitor/stem cell proliferation(Black, Mazerolle et al. 2003; Moshiri and Reh 2004). The overproliferation in the progenitor/stem cell populations of the juvenile *ptc2*$^{-/-}$ retina certainly supports this role. While, to our knowledge, no histological characterizations of human BCNS retinas have been published to date, the results presented here could provide valuable insight into the contributing causes of visual impairment in BCNS patients. Whether *ptc2*$^{-/-}$ juvenile fish possess BCNS-related phenotypes in tissues other than the retina remains to be investigated; however, the utility

of the *ptc2*$^{-/-}$ mutant line for such studies provides an additional, and valuable, *in vivo* model for the study of Patched function during development and in diseases like BCNS.

CHAPTER IV

Future Directions

IV.1 INVESTIGATING THE MOLECULAR MECHANISMS UNDERLYING PATCHED2-DEPENDENT CONTROL OF RETINAL PROLIFERATION

The role of the Hedgehog pathway in retinogenesis has been studied extensively, with multiple reports establishing its importance in both the proliferation (Jensen and Wallace 1997; Moshiri, McGuire et al. 2005; Locker, Agathocleous et al. 2006) and differentiation (Shkumatava, Fischer et al. 2004; Masai, Yamaguchi et al. 2005) of retinal progenitors. Studies in *Xenopus* and zebrafish have shown that the Hedgehog pathway controls cell cycle kinetics through the positive regulation of *cyclins B1*, *D1*, and *A2*, and the phosphatase *cdc25*, reducing the length of the G1 and G2 phases of the cell cycle (Locker, Agathocleous et al. 2006). At the same time, other findings suggest that the Hedgehog pathway controls cell cycle kinetics through the transcriptional regulation of the cyclin dependent kinase (CDK) inhibitors *p27kip1* and *p57kip2* (Dyer and Cepko 2001; Masai, Yamaguchi et al. 2005; Shkumatava and Neumann 2005). CDK inhibitors (CKIs) bind to and inhibit cyclin/CDK complexes, thereby negatively regulating cell cycle progression (Dyer and Cepko 2001). Due to the multiple roles of the Hedgehog pathway in retinal development and apparent contradictions in published results, the exact mechanisms in which this pathway controls retinogenesis are unresolved. Our characterization of the embryonic and post-embryonic *ptc2*$^{-/-}$ retina revealed that loss of Patched2 function results in increased size of the progenitor/stem cell population of the

CMZ in late embryonic and post-embryonic stages. It would therefore be of great interest to study the molecular mechanisms through which Patched2-depedent Hh activity controls the progenitor/stem cell population of the CMZ.

Gene expression studies in *Xenopus* and zebrafish have identified a peripheral-to-central spatial arrangement of cells in the CMZ the recapitulates early retinal development, and have provided a growing list of molecular markers for the study of neurogenesis in the CMZ (Raymond, Barthel et al. 2006; Stephens, Senecal et al. 2010; Xue and Harris 2011). These studies have also identified members of genetic pathways with known roles in stem cell maintenance and/or proliferation. The expression of the Notch pathway components *notch1a/b, her2/6*, and *deltaC* in the peripheral CMZ suggests a role for Notch in regulating some aspect of neurogenesis in the CMZ (Raymond, Barthel et al. 2006). In chick, *Hairy1*, a known Notch pathway effector, has been shown to act downstream of Wnt2b to maintain the retinal stem/progenitor cell population of the CMZ (Kubo and Nakagawa 2009). Additional studies in *Xenopus* have implicated the Wnt pathway in controlling the proliferation of the CMZ, and loss of *adenomatous polyposis coli (apc)*, a negative regulator of the Wnt pathway, results in an expansion of the CMZ in zebrafish (Stephens, Senecal et al. 2010), a phenotype similar to that observed in the embryonic *ptc2*$^{-/-}$ retina (Bibliowicz and Gross 2009). A comparative analysis of gene expression using available markers in *ptc2*$^{-/-}$ and sibling retinas could provide insight into the molecular mechanisms through which Patched2 controls in

proliferation in the CMZ and might prove valuable in uncovering the potential interactions between the Hh and other developmental pathways, such as Notch and Wnt.

Studies in mouse have shown that Hh regulates the transcription of *Hes1*, a bHLH transcription factor and known Notch pathway component, in a Notch-independent manner (Wall, Mears et al. 2009). Interestingly, increased retinal proliferation in *Ptch1*$^{+/-}$ mice was rescued in *Ptch1*$^{+/-}$/*Hes1*$^{+/-}$ double mutants, suggesting that Hes1 functions to control Patched-dependent regulation of retinal proliferation. However, the factors that might be controlled by Hes1 in this context have yet to be identified. Consistent with the findings in mouse, the zebrafish *Hes1* orthologue *her6* is expressed throughout the embryonic zebrafish CNS and has been shown to act downstream of Hh to control neurogenesis in the developing zebrafish spinal cord (Cunliffe 2004; Scholpp, Delogu et al. 2009). Analysis of transcriptional regulation of cell-cycle genes in *her6* morpholino-injected zebrafish (*her6*-MO) will provide insight about the mechanisms in which Hes1 controls proliferation in the retina. In addition, comparative analysis of gene expression in *ptc2*$^{-/-}$/ *her6*-MO, *ptc2*$^{-/-}$, and wild-type CMZs could reveal which aspects of Patched-dependent retinal proliferation are potentiated by *her6* transcriptional activity.

The regulation of the Notch effectors *Hes1* and *Hairy1* by the Hh and Wnt signaling pathways, respectively, in the retina suggest that Hh and Wnt might cooperate to control Notch-related molecular processes. The expansion of the CMZ stem/progenitor cell population in both the *ptc2*$^{-/-}$ and *apc* zebrafish mutants suggest that,

consistent with their known roles in the retina, these pathways might promote proliferation in this context. These effects on the size of the proliferative cell population of the CMZ can be achieved directly by controlling cell cycle dynamics or indirectly by inhibiting cell cycle exit. Indeed, reports in zebrafish and *Xenopus* have suggested that Notch activity controls both retinal proliferation and cell cycle exit (Ohnuma, Hopper et al. 2002; Yamaguchi, Tonou-Fujimori et al. 2005). Additionally, it has been proposed that modulation of Notch activity by both Notch-dependent and Notch-independent regulation might be required to control multiple functions of Notch signaling during neurogenesis, and that proliferation and differentiation are dependent on Hes1 dosage (Wall, Mears et al. 2009). Therefore, combinatorial modulation of Hh and Wnt levels using chemical and/or genetic inhibitors and activators that affect these pathways will be used. Resulting changes in the levels of Notch targets could be assayed using qRT-PCR and correlated with CMZ phenotypes, and the expression of positive and negative regulators of the cell (e.g. *cyclin D1* and *p27*, respectively). Such analyses would provide a potential explanation for the mechanism by which Notch activity in the CMZ is controlled by Hh and Wnt activity and how proliferation and/or cell cycle exit are regulated in the zebrafish CMZ.

IV.2 INVESTIGATING THE CELLULAR AND MOLECULAR MECHANISMS UNDERLYING ECTOPIC PROLIFERATION IN THE JUVENILE PATCHED2 MUTANT RETINA

IV.2.1 Cell population(s) of origin

Juvenile *ptc2*$^{-/-}$ mutants possess peripheral retinal dysplasias that include the presence of ectopic neuronal clusters in the INL, and regions of disrupted retinal lamination. Through BrdU/EdU pulse-chase and immunohistochemistry assays, we demonstrated that a population of ectopically proliferating cells give rise to the ectopic neuronal clusters in the INL of *ptc2*$^{-/-}$ mutants, and that this contributes to retinal dysplasia in the mutant eye. Our results therefore demonstrate a direct link between overproliferation and retinal dysplasia in the *ptc2*$^{-/-}$ juvenile retina and establish ectopic proliferation as the likely cellular underpinning of retinal dysplasia in juvenile *ptc2*$^{-/-}$ mutants (Bibliowicz and Gross, *submitted*). However, the cellular origin(s) of ectopic proliferation in the *ptc2*$^{-/-}$ juvenile retina are still unclear.

In order to identify the cell type(s) that give rise to ectopically proliferative cells in the *ptc2*$^{-/-}$ juvenile retina, the use of transgenic lineage-tracing zebrafish lines is necessary. We have shown, by utilizing the *gfap::GFAP* transgenic line, that the ectopic proliferation is unlikely to be derived from Müller glia. However, this approach has its limitations, since proliferative Müller glia lose certain molecular markers, such as GFAP, in response to physical injury or upon exposure to growth factors (Fischer and Reh 2003; Bernardos, Barthel et al. 2007). In response to physical injury, Müller glia that re-enter the cell cycle up-regulate *tubulin a1a* (Senut, Gulati-Leekha et al. 2004; Ramachandran, Reifler et al. 2010). Unpublished observations using *ptc2*$^{-/-}$*;tuba1a*::*CreER*^{T2}*; β-actin2::LCLG* transgenic fish suggest that Müller glia cell cycle re-entry is unlikely to underlie ectopic proliferation since no expression of GFP was observed in dysplasic regions of the *ptc2*$^{-/-}$*;tuba1a*::*CreER*^{T2}*; β-actin2::LCLG* juvenile retina.

The generation of additional transgenic lineage tracing lines that express fluorescent reporters in candidate cell populations of origin and their progeny could provide evidence of the cellular origin of ectopic proliferation in the dysplasic *ptc2*$^{-/-}$ juvenile retina. A *gfap*::*CreER*^{T2}*; β-actin2::LCLG* transgenic line would permanently label Müller glia and their progeny, while progenitors derived from other cell types could also be traced by utilizing cell type-specific promoters to drive Cre expression. To date, no molecular markers that are expressed exclusively in the retinal stem/progenitor cell population of the CMZ have been described, making lineage tracing of CMZ-derived progenitors difficult. However, the continual development and improvement of Cre/Lox-based transgenic tools for lineage tracing in zebrafish (Thummel, Burket et al. 2005; Hans, Freudenreich et al. 2011) and the future discovery of CMZ-specific molecular markers might enable such experiments in the future.

While our data support the possibility that ectopic neurons in the *ptc2*$^{-/-}$ juvenile retina arise from an ectopic proliferative event originating in a stem/progenitor cell population, other cells of origin are possible. Studies utilizing a mouse model for retinoblastoma, a rare childhood retinal tumor, have suggested that tumorigenesis results from the clonal expansion of the differentiated horizontal cells that re-enter the cell cycle as a result of mutations in the *p107* gene (Ajioka, Martins et al. 2007). In addition, loss of function of the cell cycle inhibitors *p19* and *p27* has supported the susceptibility of differentiated horizontal cells to re-enter the cell cycle, as *p19;p27*-deficient mice contained proliferative horizontal cells (Cunningham, Levine et al. 2002). Since the ectopic neuronal clusters observed in *ptc2*$^{-/-}$ retinas contain multiple cell types, clonal expansion of a differentiated cells seems unlikely to be the underlying cause of retinal dysplasia in this mutant. However, it is formally possible that de-differentiation or

transdifferentiation of a retinal neuron could occur as a result of the loss of Patched2 function. To test this possibility a BrdU incorporation assay could be combined with immunohistochemical analysis using the horizontal marker Calbindin (Rohrenbeck, Wassle et al. 1987) to identify differentiated horizontal cells that might be inappropriately proliferating in the *ptc2*$^{-/-}$ juvenile retina. In the case that initial experiments support this possibility, the Cre/Lox approach outlined above can be carried out to lineage trace horizontal cells and their progeny.

IV.2.1 Cell migration and the cell cycle

Proper lamination in the embryonic retina has been shown to be dependent on the establishment of apical-basal polarity (see section I.2). The nuclei of proliferative progenitors migrate as they progress through the cell cycle. Specific phases of the cell cycle take place in specific regions of the proliferative neuroepithelium, with M-phase occurring at the apical surface and S-phase in the basal (vitreal). Abnormal migration of progenitor nuclei can result in improper cell cycle progression and inappropriate differentiation, and mutations affecting both cell polarity and migration have been shown to result in disrupted retinal lamination of the embryonic zebrafish eye (Del Bene, Wehman et al. 2008; Yamaguchi, Imai et al. 2010). In zebrafish mutants in which cell polarity is disrupted, defective neurogenesis and hyperplasia have been linked to disruptions in Hh and Notch signaling (Yamaguchi, Imai et al. 2010). Localized disruptions in polarity and/or migration of CMZ-derived progenitors could therefore potentially result in the retinal dysplasias observed in *ptc2*$^{-/-}$ mutant retina. Analysis of the location of cell cycle progression utilizing markers of specific phase of the cell cycle

within the *ptc2*$^{-/-}$ CMZ (e.g. phosphohistone H3 for M-phase and BrdU for S-phase) might prove helpful in beginning to test this possibility.

The regulation of cell motility and migration has also been linked directly to the activity cell cycle regulators. *p57*^{kip2}, which is transcriptionally regulated by the Shh in the developing zebrafish retina (Shkumatava and Neumann 2005), has been shown to regulate the activity of the actin depolymerization factor cofilin indirectly by binding to LIM-kinase 1 (Yokoo, Toyoshima et al. 2003; Vlachos and Joseph 2009). *p27*^{kip1} binds to RhoA, a regulator of the actin cytoskeleton, to influence cell migration in the mouse cerebral cortex (Nguyen, Besson et al. 2006). These findings highlight a novel role for cell cycle regulators in influencing actin dynamics and raise the possibility that defective cell migration can be an additional contributing factor to the retinal dysplasias in the *ptc2*$^{-/-}$ juvenile retina. Consistent with this possibility, Hh signaling has recently been shown to promote the migration of retinal stem cells from the adult mouse subventriular zone to the olfactory bulb (Angot, Loulier et al. 2008). The analysis of cell migratory and cytoskeletal dynamics via *in vivo* imaging in the *ptc2*$^{-/-}$ retina could provide interesting and novel insights into the potential role of Hh activity on cell migratory and cytoskeletal dynamics.

CONCLUDING REMARKS

Since its first characterization as a segment polarity gene in *Drosophila* (Perrimon and Mahowald 1987), the importance of the Hh pathway has been highlighted by its conserved roles in the CNS that extend from patterning to proliferation, neuronal migration, and axon growth (Marti and Bovolenta 2002). Numerous studies have contributed to our understanding of its multiple roles in the developing and adult retina. The work presented here sheds new light on the function of the Hh receptor *ptc2* in controlling the proliferation of the late- and post-embryonic retinal stem/progenitor cells and the possible implications of loss of Ptch function in the context of retinal disease. Future studies will build on this work to further investigate the molecular mechanisms underlying Ptch-dependent regulation of retinal proliferation with the hope of contributing to our understanding of the cellular and molecular underpinnings of human ocular disorders, such as those found in human patients suffering from BCNS.

APPENDIX A
Materials and Methods

A.1 ZEBRAFISH MAINTENANCE AND STRAINS

Zebrafish (*Danio rerio*) were maintained at 28.5C on a 14h light/10h dark cycle. Embryos were obtained from the natural spawning of heterozygous carriers set up in pair-wise crosses. *ptc2*^{tj222} and *Tg(gfap:GFP)mi200* outcrosses were obtained from the Zebrafish International Resource Center and were propagated by repeated outcrosses to AB fish. All animals were treated in accordance with provisions established at the University of Texas at Austin governing animal use and care and adhere to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Genotyping of *ptc2*^{tj222} juvenile fish was performed by isolating genomic DNA as previously described (Westerfield 2000), and amplifying the genomic region containing the mutation by PCR using the following primer set: Forward: 5'-ggcagtggtggtggtgtttaac-3' and Reverse: 5'-cgagcctttatttagccagttg-3'. Sequencing was then performed on single fish using the reverse primer to identify individuals containing the mutation (Koudijs, den Broeder et al. 2005).

A.2 HISTOLOGY

Histology was performed as described in (Nuckels and Gross 2007). Briefly, embryos or 6 week old juvenile zebrafish were euthanized and fixed for two days at 4°C in a solution of 1% (w/v) paraformaldehyde (PFA), 2.5% glutaraldehyde and 3% sucrose in PBS/2% OsO_4 solution. They were washed 3x5 min in PBS at room temperature and further dehydrated 2x10 min in propylene oxide, and then infiltrated 1-2h in a 50% propylene oxide/50% Epon/Araldite mixture (Polysciences, Inc.). Samples were then incubated overnight at RT in 100% Epon/Araldite resin with caps open to allow for propylene oxide evaporation and resin infiltration, they were embedded and baked at 60°C for 2-3 days. 1μm sections were cut, mounted on glass slides and stained in a 1% methylene blue/1% borax solution. Sections were mounted in DPX (Electron Microscopy Sciences) and photographed on a Leica DMRB microscope mounted with a DFC320 digital camera.

A.3 IMMUNOHISTOCHEMISTRY

Immunohistochemistry was performed as described in (Uribe and Gross 2007). Fish (embryos or 6 week old juveniles) were collected and fixed overnight at 4°C in a 4% PFA solution in PBS. Fish were then washed 3X10 min in PBS and incubated in a 25% sucrose/PBS solution for 1h followed by 35% sucrose/PBS for 1h. Fish were mounted in Tissue Freezing Medium (Triangle Biomedical Sciences, Inc.) and immediately transferred to -80°C freezer for storage. Frozen blocks were sectioned at 12μm, mounted

on gelatin coated slides, and let dry for 2h. Slides were then re-hydrated in PBTD [0.1% Tween, 1% DMSO in 1x PBS] and then blocked using 5%NGS/PBTD for 2h. Slides were incubated in primary antibody diluted in block in a humid chamber over-night at 4°C. When necessary, nuclear staining was obtained by applying Sytox-Green (Molecular Probes) diluted 1:10,000 in block immediately after removal of primary antibody. Slides were washed 3x10min with PBTD and then applied with secondary antibody in block for 1h. After 3x10min washes in PBTD, slides were mounted with Vectashield (Vector Laboratories, Inc.) and coverslipped. Samples were stored at 4°C for up to one week and imaged on a Zeiss LSM5 Pascal laser scanning confocal microscope. The following primary antibodies were used: bipolar cells (PKC, 1:200, Santa Cruz Biotechnology), Müller glia (GFAP-zrf1, 1:100, ZIRC and GS, 1:500, Millipore), rods (zpr3, 1:100, ZIRC), green/red cones (zpr1, 1:100, ZIRC), PH3 (1:200, Upstate), anti-Islet1 (39.4D5, Developmental Studies Hybridoma Bank, 1:10). Blue opsin (1:500) and UV opsin (1:1,000) antibodies were provided by David Hyde (University of Notre Dame). Amacrine cell antibody (5E11, 1:100) was the gift of Jim Fadool. For embryonic cell counts, positively stained cells and nuclei were counted in four eye sections obtained from different embryos. For total cell counts at 33hpf, Sytox-Green staining was used. Averages were analyzed and compared using a Student's t-test.

A.4 *IN-SITU* HYBRIDIZATION

Whole mount hybridizations on zebrafish embryos were performed as in (Jowett and Lettice 1994) and hybridizations on juvenile retinal tissue sections were performed as described (Barthel and Raymond 2000) using digoxigenin labeled antisense RNA probes. The *ptc2* cDNA construct was provided by Brian Perkins (Texas A&M University).

A.5 BRDU AND EDU INCORPORATION ASSAYS

For embryonic BrdU incorporation, fish were dechorionated and incubated in fish water with 10μM 5-Bromo-2-deoxyuridine (BrdU, Sigma) and 15% DMSO at 4°C for mentioned time periods and either immediately sacrificed or washed three times in fish water and grown at 28.5°C prior to sacrifice after (Masai, Yamaguchi et al. 2005). Embryos were processed for immunohistochemistry as in (Lee, Willer et al. 2008). Mouse anti-BrdU was used at a 1:50 dilution and Cy3 anti-mouse secondary at a 1:200 dilution. Nuclei were counterstained with Sytox:Green (1:10,000, Molecular Probes). For cell counts, Brdu-positive cells and nuclei were counted in four eye sections obtained from different embryos. Averages were analyzed and compared using a Student's t-test.

For juvenile 5-Bromo-2-deoxyuridine (BrdU) incorporation assays, fish were incubated in fish water containing 5μM BrdU (Abcam) for 2 hours, fixed and prepared for immunohistochemistry as above. Prior to the blocking step, slides were incubated in 2N HCl at 37C for 20 minutes. Rat anti-BrdU (Sigma) was used at a 1:250 dilution. For

cell counts, positively stained cells and nuclei were counted from three consecutive sections for each individual. Averages were analyzed and compared using a Student's t-test.

For juvenile BrdU/EdU 'pulse-chase-pulse' experiments, fish were exposed to BrdU for 8 hours and then returned to tanks for two days. EdU incorporation was then performed before fish were fixed and prepared for immunohistochemistry. For EdU incorporation, a solution of 400uM 5-ethynyl-2'-deoxyuridine (Invitrogen) in PBS was prepared from a stock of 10mM EdU in DMSO. 100nL of EdU solution was injected into each eye through the cornea using a glass injection needle. Fish were allowed to recover for two hours in fish water before fixation. EdU detection was performed according to the manufacturer's protocol (Click-iT, Invitrogen), followed by HCl treatment and subsequent immunohistochemical detection of BrdU.

A.6 FLUORESCENCE ACTIVATED CELL SORTING (FACS) AND QUANTITATIVE REAL-TIME PCR (QRT-PCR)

Isolation of Müller glia from 6 week old juvenile *Tg(gfap:GFP)mi2001* retinas was performed as outlined in (Qin, Barthel et al. 2009), and carried out on an FACSAria cell sorter (BD Biosciences). Three retinas, obtained from three different individuals, were pooled to make up each sample. RNA was extracted using RNAqeous-Micro (Ambion) according to the manufacturer's protocol. Total RNA was reverse-transcribed

using iScript cDNA Synthesis Kit (Bio-Rad) and qRT-PCR performed using the Power SYBR Green kit (Applied Biosystems) and performed on the Applied Biosystems 7900HT. To determine expression levels, the standard curve method was used, relative to both *beta-actin* and *gpia* (*glucose phosphate isomerase a* (Qin, Barthel et al. 2009)), yielding similar results. The entire experiment was performed three times and data were analyzed for significance using a Student's t-test. Primer pairs used for qRT-PCR analysis were as follows:

ptc2- Forward: 5'-ctccattcctgccagcagac-3' Reverse: 5'-ctcgatggcctccacgaac-3'

beta-actin- Forward: 5'-ccgtgacatcaaggagaagct-3' Reverse: 5'-tcgtggataccgcaagattcc-3'

gpia- Forward: 5'-tccaaggaaacaagccaagca-3' Reverse: 5'-ttccacatcacaccctgcac-3'

References

Agathocleous, M. and W. A. Harris (2009). "From progenitors to differentiated cells in the vertebrate retina." Annual review of cell and developmental biology **25**: 45-69.

Agathocleous, M., I. Iordanova, et al. (2009). "A directional Wnt/beta-catenin-Sox2-proneural pathway regulates the transition from proliferation to differentiation in the Xenopus retina." Development **136**(19): 3289-3299.

Ahn, S. and A. L. Joyner (2005). "In vivo analysis of quiescent adult neural stem cells responding to Sonic hedgehog." Nature **437**(7060): 894-897.

Ajioka, I., R. A. Martins, et al. (2007). "Differentiated horizontal interneurons clonally expand to form metastatic retinoblastoma in mice." Cell **131**(2): 378-390.

Amato, M. A., S. Boy, et al. (2004). "Hedgehog signaling in vertebrate eye development: a growing puzzle." Cell Mol Life Sci **61**(7-8): 899-910.

Amirpour, N., F. Karamali, et al. (2011). "Differentiation of Human Embryonic Stem Cell-Derived Retinal Progenitors into Retinal Cells by Sonic Hedgehog and/or Retinal Pigmented Epithelium and Transplantation into the Subretinal Space of Sodium Iodate-Injected Rabbits." Stem cells and development.

Angot, E., K. Loulier, et al. (2008). "Chemoattractive activity of sonic hedgehog in the adult subventricular zone modulates the number of neural precursors reaching the olfactory bulb." Stem cells **26**(9): 2311-2320.

Austin, C. P., D. E. Feldman, et al. (1995). "Vertebrate retinal ganglion cells are selected from competent progenitors by the action of Notch." Development **121**(11): 3637-3650.

Bao, Z. Z. and C. L. Cepko (1997). "The expression and function of Notch pathway genes in the developing rat eye." The Journal of neuroscience : the official journal of the Society for Neuroscience **17**(4): 1425-1434.

Barthel, L. K. and P. A. Raymond (2000). "In situ hybridization studies of retinal neurons." Methods in enzymology **316**: 579-590.

Baye, L. M. and B. A. Link (2008). "Nuclear migration during retinal development." Brain research **1192**: 29-36.

Bernardos, R. L., L. K. Barthel, et al. (2007). "Late-stage neuronal progenitors in the retina are radial Muller glia that function as retinal stem cells." The Journal of neuroscience : the official journal of the Society for Neuroscience **27**(26): 7028-7040.

Bernardos, R. L., L. K. Barthel, et al. (2007). "Late-stage neuronal progenitors in the retina are radial Muller glia that function as retinal stem cells." J Neurosci **27**(26): 7028-7040.

Bernardos, R. L., S. I. Lentz, et al. (2005). "Notch-Delta signaling is required for spatial patterning and Muller glia differentiation in the zebrafish retina." Developmental biology **278**(2): 381-395.

Bernardos, R. L. and P. A. Raymond (2006). "GFAP transgenic zebrafish." Gene expression patterns : GEP **6**(8): 1007-1013.

Bibliowicz, J. and J. M. Gross (2009). "Expanded progenitor populations, vitreo-retinal abnormalities, and Muller glial reactivity in the zebrafish leprechaun/patched2 retina." BMC developmental biology **9**: 52.

Bibliowicz, J., R. K. Tittle, et al. (2011). "Toward a better understanding of human eye disease insights from the zebrafish, Danio rerio." Progress in molecular biology and translational science **100**: 287-330.

Bijlsma, M. F., C. A. Spek, et al. (2006). "Repression of smoothened by patched-dependent (pro-)vitamin D3 secretion." PLoS biology **4**(8): e232.

Bilotta, J. and S. Saszik (2001). "The zebrafish as a model visual system." Int J Dev Neurosci **19**(7): 621-629.

Black, G. C., C. J. Mazerolle, et al. (2003). "Abnormalities of the vitreoretinal interface caused by dysregulated Hedgehog signaling during retinal development." Human molecular genetics **12**(24): 3269-3276.

Black, G. C., C. J. Mazerolle, et al. (2003). "Abnormalities of the vitreoretinal interface caused by dysregulated Hedgehog signaling during retinal development." Hum Mol Genet **12**(24): 3269-3276.

Bringmann, A., T. Pannicke, et al. (2006). "Muller cells in the healthy and diseased retina." Prog Retin Eye Res **25**(4): 397-424.

Cameron, D. A. (2000). "Cellular proliferation and neurogenesis in the injured retina of adult zebrafish." Vis Neurosci **17**(5): 789-797.

Casali, A. and G. Struhl (2004). "Reading the Hedgehog morphogen gradient by measuring the ratio of bound to unbound Patched protein." Nature **431**(7004): 76-80.

Casarosa, S., M. A. Amato, et al. (2003). "Xrx1 controls proliferation and multipotency of retinal progenitors." Molecular and cellular neurosciences **22**(1): 25-36.

Cepko, C. L., C. P. Austin, et al. (1996). "Cell fate determination in the vertebrate retina." Proceedings of the National Academy of Sciences of the United States of America **93**(2): 589-595.

Chen, J. K., J. Taipale, et al. (2002). "Inhibition of Hedgehog signaling by direct binding of cyclopamine to Smoothened." Genes & development **16**(21): 2743-2748.

Chiang, C., Y. Litingtung, et al. (1996). "Cyclopia and defective axial patterning in mice lacking Sonic hedgehog gene function." Nature **383**(6599): 407-413.

Clark, A. M., S. Yun, et al. (2008). "Negative regulation of Vsx1 by its paralog Chx10/Vsx2 is conserved in the vertebrate retina." Brain research **1192**: 99-113.

Cooper, M. K., J. A. Porter, et al. (1998). "Teratogen-mediated inhibition of target tissue response to Shh signaling." Science **280**(5369): 1603-1607.

Cunliffe, V. T. (2004). "Histone deacetylase 1 is required to repress Notch target gene expression during zebrafish neurogenesis and to maintain the production of motoneurones in response to hedgehog signalling." Development **131**(12): 2983-2995.

Cunningham, J. J., E. M. Levine, et al. (2002). "The cyclin-dependent kinase inhibitors p19(Ink4d) and p27(Kip1) are coexpressed in select retinal cells and act cooperatively to control cell cycle exit." Molecular and cellular neurosciences **19**(3): 359-374.

Dahmane, N. and A. Ruiz i Altaba (1999). "Sonic hedgehog regulates the growth and patterning of the cerebellum." Development **126**(14): 3089-3100.

Dakubo, G. D., C. Mazerolle, et al. (2008). "Indian hedgehog signaling from endothelial cells is required for sclera and retinal pigment epithelium development in the mouse eye." Dev Biol **320**(1): 242-255.

Das, A. V., K. B. Mallya, et al. (2006). "Neural stem cell properties of Muller glia in the mammalian retina: regulation by Notch and Wnt signaling." Developmental biology **299**(1): 283-302.

Davies, J. P., F. W. Chen, et al. (2000). "Transmembrane molecular pump activity of Niemann-Pick C1 protein." Science **290**(5500): 2295-2298.

Del Bene, F., A. M. Wehman, et al. (2008). "Regulation of neurogenesis by interkinetic nuclear migration through an apical-basal notch gradient." Cell **134**(6): 1055-1065.

Denayer, T., M. Locker, et al. (2008). "Canonical Wnt signaling controls proliferation of retinal stem/progenitor cells in postembryonic Xenopus eyes." Stem cells **26**(8): 2063-2074.

Dessaud, E., L. L. Yang, et al. (2007). "Interpretation of the sonic hedgehog morphogen gradient by a temporal adaptation mechanism." Nature **450**(7170): 717-720.

Dorsky, R. I., W. S. Chang, et al. (1997). "Regulation of neuronal diversity in the Xenopus retina by Delta signalling." Nature **385**(6611): 67-70.

Dowling, J. E. (1987). The Retina. Cambridge, MA, Harvard University Press.

Dyer, M. A. and C. L. Cepko (2000). "Control of Muller glial cell proliferation and activation following retinal injury." Nat Neurosci **3**(9): 873-880.

Dyer, M. A. and C. L. Cepko (2000). "p57(Kip2) regulates progenitor cell proliferation and amacrine interneuron development in the mouse retina." Development **127**(16): 3593-3605.

Dyer, M. A. and C. L. Cepko (2001). "p27Kip1 and p57Kip2 regulate proliferation in distinct retinal progenitor cell populations." J Neurosci **21**(12): 4259-4271.

Dyer, M. A. and C. L. Cepko (2001). "Regulating proliferation during retinal development." Nature reviews. Neuroscience **2**(5): 333-342.

Dyer, M. A., F. J. Livesey, et al. (2003). "Prox1 function controls progenitor cell proliferation and horizontal cell genesis in the mammalian retina." Nature genetics **34**(1): 53-58.

Easter, S. S., Jr. and G. N. Nicola (1996). "The development of vision in the zebrafish (Danio rerio)." Dev Biol **180**(2): 646-663.

Ekker, S. C., A. R. Ungar, et al. (1995). "Patterning activities of vertebrate hedgehog proteins in the developing eye and brain." Curr Biol **5**(8): 944-955.

Ekker, S. C., A. R. Ungar, et al. (1995). "Patterning activities of vertebrate hedgehog proteins in the developing eye and brain." Current biology : CB **5**(8): 944-955.

Ericson, J., J. Muhr, et al. (1995). "Sonic hedgehog induces the differentiation of ventral forebrain neurons: a common signal for ventral patterning within the neural tube." Cell **81**(5): 747-756.

Ericson, J., P. Rashbass, et al. (1997). "Pax6 controls progenitor cell identity and neuronal fate in response to graded Shh signaling." Cell **90**(1): 169-180.

Fadool, J. M. and J. E. Dowling (2008). "Zebrafish: a model system for the study of eye genetics." Prog Retin Eye Res **27**(1): 89-110.

Fausett, B. V. and D. Goldman (2006). "A role for alpha1 tubulin-expressing Muller glia in regeneration of the injured zebrafish retina." J Neurosci **26**(23): 6303-6313.

Fischer, A. J. and T. A. Reh (2003). "Potential of Muller glia to become neurogenic retinal progenitor cells." Glia **43**(1): 70-76.

Frank-Kamenetsky, M., X. M. Zhang, et al. (2002). "Small-molecule modulators of Hedgehog signaling: identification and characterization of Smoothened agonists and antagonists." Journal of biology **1**(2): 10.

Fuccillo, M., A. L. Joyner, et al. (2006). "Morphogen to mitogen: the multiple roles of hedgehog signalling in vertebrate neural development." Nat Rev Neurosci **7**(10): 772-783.

Fuccillo, M., A. L. Joyner, et al. (2006). "Morphogen to mitogen: the multiple roles of hedgehog signalling in vertebrate neural development." Nature reviews. Neuroscience **7**(10): 772-783.

Furukawa, T., S. Mukherjee, et al. (2000). "rax, Hes1, and notch1 promote the formation of Muller glia by postnatal retinal progenitor cells." Neuron **26**(2): 383-394.

Gabriel, R., M. Wilhelm, et al. (1993). "Morphology and distribution of Muller cells in the retina of the toad Bufo marinus." Cell Tissue Res **272**(1): 183-192.

Goldsmith, P. and W. A. Harris (2003). "The zebrafish as a tool for understanding the biology of visual disorders." Semin Cell Dev Biol **14**(1): 11-18.

Goldsmith, P. and W. A. Harris (2003). "The zebrafish as a tool for understanding the biology of visual disorders." Seminars in cell & developmental biology **14**(1): 11-18.

Goodrich, L. V., L. Milenkovic, et al. (1997). "Altered neural cell fates and medulloblastoma in mouse patched mutants." Science **277**(5329): 1109-1113.

Green, E. S., J. L. Stubbs, et al. (2003). "Genetic rescue of cell number in a mouse model of microphthalmia: interactions between Chx10 and G1-phase cell cycle regulators." Development **130**(3): 539-552.

Gross, J. M. and B. D. Perkins (2008). "Zebrafish mutants as models for congenital ocular disorders in humans." Mol Reprod Dev **75**(3): 547-555.

Gross, J. M., B. D. Perkins, et al. (2005). "Identification of zebrafish insertional mutants with defects in visual system development and function." Genetics **170**(1): 245-261.

Hahn, H., J. Christiansen, et al. (1996). "A mammalian patched homolog is expressed in target tissues of sonic hedgehog and maps to a region associated with developmental abnormalities." The Journal of biological chemistry **271**(21): 12125-12128.

Hahn, H., C. Wicking, et al. (1996). "Mutations of the human homolog of Drosophila patched in the nevoid basal cell carcinoma syndrome." Cell **85**(6): 841-851.
Hans, S., D. Freudenreich, et al. (2011). "Generation of a non-leaky heat shock-inducible Cre line for conditional Cre/lox strategies in zebrafish." Developmental dynamics : an official publication of the American Association of Anatomists **240**(1): 108-115.
Harada, C., Y. Mitamura, et al. (2006). "The role of cytokines and trophic factors in epiretinal membranes: involvement of signal transduction in glial cells." Prog Retin Eye Res **25**(2): 149-164.
Hartong, D. T., E. L. Berson, et al. (2006). "Retinitis pigmentosa." Lancet **368**(9549): 1795-1809.
Heisenberg, C. P., M. Brand, et al. (1996). "Genes involved in forebrain development in the zebrafish, Danio rerio." Development **123**: 191-203.
Hidalgo, A. and P. Ingham (1990). "Cell patterning in the Drosophila segment: spatial regulation of the segment polarity gene patched." Development **110**(1): 291-301.
High, A. and W. Zedan (2005). "Basal cell nevus syndrome." Current opinion in oncology **17**(2): 160-166.
Ingham, P. W. (1998). "The patched gene in development and cancer." Current opinion in genetics & development **8**(1): 88-94.
Ingham, P. W. and A. P. McMahon (2001). "Hedgehog signaling in animal development: paradigms and principles." Genes & development **15**(23): 3059-3087.
Jensen, A. M. and V. A. Wallace (1997). "Expression of Sonic hedgehog and its putative role as a precursor cell mitogen in the developing mouse retina." Development **124**(2): 363-371.
Jiang, J. and C. C. Hui (2008). "Hedgehog signaling in development and cancer." Developmental cell **15**(6): 801-812.
Johnson, R. L., L. Milenkovic, et al. (2000). "In vivo functions of the patched protein: requirement of the C terminus for target gene inactivation but not Hedgehog sequestration." Mol Cell **6**(2): 467-478.
Johnson, R. L., L. Milenkovic, et al. (2000). "In vivo functions of the patched protein: requirement of the C terminus for target gene inactivation but not Hedgehog sequestration." Molecular cell **6**(2): 467-478.
Johnson, R. L., A. L. Rothman, et al. (1996). "Human homolog of patched, a candidate gene for the basal cell nevus syndrome." Science **272**(5268): 1668-1671.
Johnson, S. L. and J. A. Weston (1995). "Temperature-sensitive mutations that cause stage-specific defects in Zebrafish fin regeneration." Genetics **141**(4): 1583-1595.
Jowett, T. and L. Lettice (1994). "Whole-mount in situ hybridizations on zebrafish embryos using a mixture of digoxigenin- and fluorescein-labelled probes." Trends in genetics : TIG **10**(3): 73-74.
Julian, D., K. Ennis, et al. (1998). "Birth and fate of proliferative cells in the inner nuclear layer of the mature fish retina." The Journal of comparative neurology **394**(3): 271-282.

Julian, D., K. Ennis, et al. (1998). "Birth and fate of proliferative cells in the inner nuclear layer of the mature fish retina." J Comp Neurol **394**(3): 271-282.
Karlstrom, R. O., W. S. Talbot, et al. (1999). "Comparative synteny cloning of zebrafish you-too: mutations in the Hedgehog target gli2 affect ventral forebrain patterning." Genes Dev **13**(4): 388-393.
Kassen, S. C., V. Ramanan, et al. (2007). "Time course analysis of gene expression during light-induced photoreceptor cell death and regeneration in albino zebrafish." Dev Neurobiol **67**(8): 1009-1031.
Koudijs, M. J., M. J. den Broeder, et al. (2008). "Genetic analysis of the two zebrafish patched homologues identifies novel roles for the hedgehog signaling pathway." BMC Dev Biol **8**: 15.
Koudijs, M. J., M. J. den Broeder, et al. (2005). "The zebrafish mutants dre, uki, and lep encode negative regulators of the hedgehog signaling pathway." PLoS Genet **1**(2): e19.
Koudijs, M. J., M. J. den Broeder, et al. (2005). "The zebrafish mutants dre, uki, and lep encode negative regulators of the hedgehog signaling pathway." PLoS genetics **1**(2): e19.
Kubo, F. and S. Nakagawa (2009). "Hairy1 acts as a node downstream of Wnt signaling to maintain retinal stem cell-like progenitor cells in the chick ciliary marginal zone." Development **136**(11): 1823-1833.
Kubo, F., M. Takeichi, et al. (2005). "Wnt2b inhibits differentiation of retinal progenitor cells in the absence of Notch activity by downregulating the expression of proneural genes." Development **132**(12): 2759-2770.
Lai, K., B. K. Kaspar, et al. (2003). "Sonic hedgehog regulates adult neural progenitor proliferation in vitro and in vivo." Nature neuroscience **6**(1): 21-27.
Lee, J., J. R. Willer, et al. (2008). "Zebrafish blowout provides genetic evidence for Patched1-mediated negative regulation of Hedgehog signaling within the proximal optic vesicle of the vertebrate eye." Dev Biol **319**(1): 10-22.
Levine, E. M., J. Close, et al. (2000). "p27(Kip1) regulates cell cycle withdrawal of late multipotent progenitor cells in the mammalian retina." Developmental biology **219**(2): 299-314.
Lewis, K. E., J. P. Concordet, et al. (1999). "Characterisation of a second patched gene in the zebrafish Danio rerio and the differential response of patched genes to Hedgehog signalling." Developmental biology **208**(1): 14-29.
Lewis, K. E., J. P. Concordet, et al. (1999). "Characterisation of a second patched gene in the zebrafish Danio rerio and the differential response of patched genes to Hedgehog signalling." Dev Biol **208**(1): 14-29.
Li, Z., M. Hu, et al. (2000). "Modulation of cell proliferation in the embryonic retina of zebrafish (Danio rerio)." Dev Dyn **219**(3): 391-401.
Link, B. A., J. M. Fadool, et al. (2000). "The zebrafish young mutation acts non-cell-autonomously to uncouple differentiation from specification for all retinal cells." Development **127**(10): 2177-2188.

Lister, J. A. (2002). "Development of pigment cells in the zebrafish embryo." Microsc Res Tech **58**(6): 435-441.
Livesey, F. J. and C. L. Cepko (2001). "Vertebrate neural cell-fate determination: lessons from the retina." Nature reviews. Neuroscience **2**(2): 109-118.
Locker, M., M. Agathocleous, et al. (2006). "Hedgehog signaling and the retina: insights into the mechanisms controlling the proliferative properties of neural precursors." Genes Dev **20**(21): 3036-3048.
Locker, M., M. Agathocleous, et al. (2006). "Hedgehog signaling and the retina: insights into the mechanisms controlling the proliferative properties of neural precursors." Genes & development **20**(21): 3036-3048.
Locker, M., C. Borday, et al. (2009). "Stemness or not stemness? Current status and perspectives of adult retinal stem cells." Current stem cell research & therapy **4**(2): 118-130.
Ma, C., D. Papermaster, et al. (1998). "A unique pattern of photoreceptor degeneration in cyclin D1 mutant mice." Proceedings of the National Academy of Sciences of the United States of America **95**(17): 9938-9943.
Machold, R., S. Hayashi, et al. (2003). "Sonic hedgehog is required for progenitor cell maintenance in telencephalic stem cell niches." Neuron **39**(6): 937-950.
Marigo, V., R. A. Davey, et al. (1996). "Biochemical evidence that patched is the Hedgehog receptor." Nature **384**(6605): 176-179.
Marquardt, T., R. Ashery-Padan, et al. (2001). "Pax6 is required for the multipotent state of retinal progenitor cells." Cell **105**(1): 43-55.
Marti, E. and P. Bovolenta (2002). "Sonic hedgehog in CNS development: one signal, multiple outputs." Trends in neurosciences **25**(2): 89-96.
Marti, E. and P. Bovolenta (2002). "Sonic hedgehog in CNS development: one signal, multiple outputs." Trends Neurosci **25**(2): 89-96.
Martinez-Morales, J. R., F. Del Bene, et al. (2005). "Differentiation of the vertebrate retina is coordinated by an FGF signaling center." Developmental cell **8**(4): 565-574.
Masai, I., D. L. Stemple, et al. (2000). "Midline signals regulate retinal neurogenesis in zebrafish." Neuron **27**(2): 251-263.
Masai, I., M. Yamaguchi, et al. (2005). "The hedgehog-PKA pathway regulates two distinct steps of the differentiation of retinal ganglion cells: the cell-cycle exit of retinoblasts and their neuronal maturation." Development **132**(7): 1539-1553.
McFarlane, S., M. E. Zuber, et al. (1998). "A role for the fibroblast growth factor receptor in cell fate decisions in the developing vertebrate retina." Development **125**(20): 3967-3975.
McLellan, J. S., S. Yao, et al. (2006). "Structure of a heparin-dependent complex of Hedgehog and Ihog." Proc Natl Acad Sci U S A **103**(46): 17208-17213.
McLellan, J. S., X. Zheng, et al. (2008). "The mode of Hedgehog binding to Ihog homologues is not conserved across different phyla." Nature **455**(7215): 979-983.
Moshiri, A., J. Close, et al. (2004). "Retinal stem cells and regeneration." The International journal of developmental biology **48**(8-9): 1003-1014.

Moshiri, A., C. R. McGuire, et al. (2005). "Sonic hedgehog regulates proliferation of the retinal ciliary marginal zone in posthatch chicks." Developmental dynamics : an official publication of the American Association of Anatomists **233**(1): 66-75.

Moshiri, A., C. R. McGuire, et al. (2005). "Sonic hedgehog regulates proliferation of the retinal ciliary marginal zone in posthatch chicks." Dev Dyn **233**(1): 66-75.

Moshiri, A. and T. A. Reh (2004). "Persistent progenitors at the retinal margin of ptc+/- mice." The Journal of neuroscience : the official journal of the Society for Neuroscience **24**(1): 229-237.

Moshiri, A. and T. A. Reh (2004). "Persistent progenitors at the retinal margin of ptc+/- mice." J Neurosci **24**(1): 229-237.

Mulieri, P. J., A. Okada, et al. (2000). "Developmental expression pattern of the cdo gene." Dev Dyn **219**(1): 40-49.

Murone, M., A. Rosenthal, et al. (1999). "Sonic hedgehog signaling by the patched-smoothened receptor complex." Curr Biol **9**(2): 76-84.

Murone, M., A. Rosenthal, et al. (1999). "Sonic hedgehog signaling by the patched-smoothened receptor complex." Current biology : CB **9**(2): 76-84.

Negishi, K., T. Teranishi, et al. (1982). "Growth zone of the juvenile goldfish retina revealed by fluorescent flat mounts." Journal of neuroscience research **7**(3): 321-330.

Neumann, C. J. (2005). "Hedgehogs as negative regulators of the cell cycle." Cell Cycle **4**(9): 1139-1140.

Neumann, C. J. and C. Nuesslein-Volhard (2000). "Patterning of the zebrafish retina by a wave of sonic hedgehog activity." Science **289**(5487): 2137-2139.

Nguyen, L., A. Besson, et al. (2006). "p27kip1 independently promotes neuronal differentiation and migration in the cerebral cortex." Genes & development **20**(11): 1511-1524.

Nickerson, P. E., J. G. Emsley, et al. (2007). "Proliferation and expression of progenitor and mature retinal phenotypes in the adult mammalian ciliary body after retinal ganglion cell injury." Investigative ophthalmology & visual science **48**(11): 5266-5275.

Nuckels, R. J. and J. M. Gross (2007). "Histological preparation of embryonic and adult zebrafish eyes." CSH protocols **2007**: pdb prot4846.

Ohnuma, S., S. Hopper, et al. (2002). "Co-ordinating retinal histogenesis: early cell cycle exit enhances early cell fate determination in the Xenopus retina." Development **129**(10): 2435-2446.

Ohnuma, S., A. Philpott, et al. (1999). "p27Xic1, a Cdk inhibitor, promotes the determination of glial cells in Xenopus retina." Cell **99**(5): 499-510.

Ohsawa, R. and R. Kageyama (2008). "Regulation of retinal cell fate specification by multiple transcription factors." Brain research **1192**: 90-98.

Otteson, D. C., A. R. D'Costa, et al. (2001). "Putative stem cells and the lineage of rod photoreceptors in the mature retina of the goldfish." Developmental biology **232**(1): 62-76.

Patel, A. and S. McFarlane (2000). "Overexpression of FGF-2 alters cell fate specification in the developing retina of Xenopus laevis." Developmental biology **222**(1): 170-180.

Peringa, J., K. M. Fung, et al. (1995). "The cellular and molecular biology of medulloblastoma." Current opinion in neurology **8**(6): 437-440.

Perrimon, N. and A. P. Mahowald (1987). "Multiple functions of segment polarity genes in Drosophila." Developmental biology **119**(2): 587-600.

Perron, M., S. Boy, et al. (2003). "A novel function for Hedgehog signalling in retinal pigment epithelium differentiation." Development **130**(8): 1565-1577.

Perron, M., S. Kanekar, et al. (1998). "The genetic sequence of retinal development in the ciliary margin of the Xenopus eye." Dev Biol **199**(2): 185-200.

Perron, M., S. Kanekar, et al. (1998). "The genetic sequence of retinal development in the ciliary margin of the Xenopus eye." Developmental biology **199**(2): 185-200.

Poss, K. D., L. G. Wilson, et al. (2002). "Heart regeneration in zebrafish." Science **298**(5601): 2188-2190.

Qin, Z., L. K. Barthel, et al. (2009). "Genetic evidence for shared mechanisms of epimorphic regeneration in zebrafish." Proc Natl Acad Sci U S A **106**(23): 9310-9315.

Qin, Z., L. K. Barthel, et al. (2009). "Genetic evidence for shared mechanisms of epimorphic regeneration in zebrafish." Proceedings of the National Academy of Sciences of the United States of America **106**(23): 9310-9315.

Ramachandran, R., A. Reifler, et al. (2010). "Conditional gene expression and lineage tracing of tuba1a expressing cells during zebrafish development and retina regeneration." The Journal of comparative neurology **518**(20): 4196-4212.

Raymond, P. A., L. K. Barthel, et al. (2006). "Molecular characterization of retinal stem cells and their niches in adult zebrafish." BMC developmental biology **6**: 36.

Raymond, P. A., L. K. Barthel, et al. (2006). "Molecular characterization of retinal stem cells and their niches in adult zebrafish." BMC Dev Biol **6**: 36.

Raymond, P. A. and P. K. Rivlin (1987). "Germinal cells in the goldfish retina that produce rod photoreceptors." Dev Biol **122**(1): 120-138.

Resnikoff, S., D. Pascolini, et al. (2004). "Global data on visual impairment in the year 2002." Bulletin of the World Health Organization **82**(11): 844-851.

Robinson, J., E. A. Schmitt, et al. (1993). "Zebrafish ultraviolet visual pigment: absorption spectrum, sequence, and localization." Proc Natl Acad Sci U S A **90**(13): 6009-6012.

Rohrenbeck, J., H. Wassle, et al. (1987). "Immunocytochemical labelling of horizontal cells in mammalian retina using antibodies against calcium-binding proteins." Neuroscience letters **77**(3): 255-260.

Sahel, J. A., D. M. Albert, et al. (1990). "[Proliferation of retinal glia and excitatory amino acids]." Ophtalmologie : organe de la Societe francaise d'ophtalmologie **4**(1): 13-16.

Sakagami, K., L. Gan, et al. (2009). "Distinct effects of Hedgehog signaling on neuronal fate specification and cell cycle progression in the embryonic mouse retina." The

Journal of neuroscience : the official journal of the Society for Neuroscience **29**(21): 6932-6944.

Scheer, N., A. Groth, et al. (2001). "An instructive function for Notch in promoting gliogenesis in the zebrafish retina." Development **128**(7): 1099-1107.

Schmitt, E. A. and J. E. Dowling (1994). "Early eye morphogenesis in the zebrafish, Brachydanio rerio." J Comp Neurol **344**(4): 532-542.

Schmitt, E. A. and J. E. Dowling (1999). "Early retinal development in the zebrafish, Danio rerio: light and electron microscopic analyses." J Comp Neurol **404**(4): 515-536.

Scholpp, S., A. Delogu, et al. (2009). "Her6 regulates the neurogenetic gradient and neuronal identity in the thalamus." Proceedings of the National Academy of Sciences of the United States of America **106**(47): 19895-19900.

Senut, M. C., A. Gulati-Leekha, et al. (2004). "An element in the alpha1-tubulin promoter is necessary for retinal expression during optic nerve regeneration but not after eye injury in the adult zebrafish." The Journal of neuroscience : the official journal of the Society for Neuroscience **24**(35): 7663-7673.

Shkumatava, A., S. Fischer, et al. (2004). "Sonic hedgehog, secreted by amacrine cells, acts as a short-range signal to direct differentiation and lamination in the zebrafish retina." Development **131**(16): 3849-3858.

Shkumatava, A. and C. J. Neumann (2005). "Shh directs cell-cycle exit by activating p57Kip2 in the zebrafish retina." EMBO Rep **6**(6): 563-569.

Shkumatava, A. and C. J. Neumann (2005). "Shh directs cell-cycle exit by activating p57Kip2 in the zebrafish retina." EMBO reports **6**(6): 563-569.

Shtutman, M., J. Zhurinsky, et al. (1999). "The cyclin D1 gene is a target of the beta-catenin/LEF-1 pathway." Proceedings of the National Academy of Sciences of the United States of America **96**(10): 5522-5527.

Soules, K. A. and B. A. Link (2005). "Morphogenesis of the anterior segment in the zebrafish eye." BMC developmental biology **5**: 12.

Soules, K. A. and B. A. Link (2005). "Morphogenesis of the anterior segment in the zebrafish eye." BMC Dev Biol **5**: 12.

Spence, J. R., J. C. Aycinena, et al. (2007). "Fibroblast growth factor-hedgehog interdependence during retina regeneration." Developmental dynamics : an official publication of the American Association of Anatomists **236**(5): 1161-1174.

Spence, J. R., M. Madhavan, et al. (2004). "The hedgehog pathway is a modulator of retina regeneration." Development **131**(18): 4607-4621.

Stenkamp, D. L. (2007). "Neurogenesis in the fish retina." International review of cytology **259**: 173-224.

Stenkamp, D. L. and R. A. Frey (2003). "Extraretinal and retinal hedgehog signaling sequentially regulate retinal differentiation in zebrafish." Developmental biology **258**(2): 349-363.

Stenkamp, D. L. and R. A. Frey (2003). "Extraretinal and retinal hedgehog signaling sequentially regulate retinal differentiation in zebrafish." Dev Biol **258**(2): 349-363.

Stenkamp, D. L., R. A. Frey, et al. (2002). "Embryonic retinal gene expression in sonic-you mutant zebrafish." Developmental dynamics : an official publication of the American Association of Anatomists **225**(3): 344-350.

Stenkamp, D. L., R. A. Frey, et al. (2002). "Embryonic retinal gene expression in sonic-you mutant zebrafish." Dev Dyn **225**(3): 344-350.

Stenkamp, D. L., R. A. Frey, et al. (2000). "Function for Hedgehog genes in zebrafish retinal development." Developmental biology **220**(2): 238-252.

Stenkamp, D. L., R. A. Frey, et al. (2000). "Function for Hedgehog genes in zebrafish retinal development." Dev Biol **220**(2): 238-252.

Stenkamp, D. L., R. Satterfield, et al. (2008). "Age-related cone abnormalities in zebrafish with genetic lesions in sonic hedgehog." Investigative ophthalmology & visual science **49**(10): 4631-4640.

Stephens, W. Z., M. Senecal, et al. (2010). "Loss of adenomatous polyposis coli (apc) results in an expanded ciliary marginal zone in the zebrafish eye." Developmental dynamics : an official publication of the American Association of Anatomists **239**(7): 2066-2077.

Stone, D. M., M. Hynes, et al. (1996). "The tumour-suppressor gene patched encodes a candidate receptor for Sonic hedgehog." Nature **384**(6605): 129-134.

Swindell, E. C., C. A. Zilinski, et al. (2008). "Regulation and function of foxe3 during early zebrafish development." Genesis **46**(3): 177-183.

Taipale, J., M. K. Cooper, et al. (2002). "Patched acts catalytically to suppress the activity of Smoothened." Nature **418**(6900): 892-897.

Takabatake, T., M. Ogawa, et al. (1997). "Hedgehog and patched gene expression in adult ocular tissues." FEBS letters **410**(2-3): 485-489.

Thummel, R., C. T. Burket, et al. (2005). "Cre-mediated site-specific recombination in zebrafish embryos." Developmental dynamics : an official publication of the American Association of Anatomists **233**(4): 1366-1377.

Trimarchi, J. M., M. B. Stadler, et al. (2008). "Individual retinal progenitor cells display extensive heterogeneity of gene expression." PloS one **3**(2): e1588.

Turner, D. L., E. Y. Snyder, et al. (1990). "Lineage-independent determination of cell type in the embryonic mouse retina." Neuron **4**(6): 833-845.

Ueno, M., K. Katayama, et al. (2006). "Cell cycle progression is required for nuclear migration of neural progenitor cells." Brain research **1088**(1): 57-67.

Uribe, R. A. and J. M. Gross (2007). "Immunohistochemistry on cryosections from embryonic and adult zebrafish eyes." Cold Spring Harbor Protocols.

van den Heuvel, M. and P. W. Ingham (1996). "smoothened encodes a receptor-like serpentine protein required for hedgehog signalling." Nature **382**(6591): 547-551.

Vihtelic, T. S., C. J. Doro, et al. (1999). "Cloning and characterization of six zebrafish photoreceptor opsin cDNAs and immunolocalization of their corresponding proteins." Vis Neurosci **16**(3): 571-585.

Vihtelic, T. S. and D. R. Hyde (2000). "Light-induced rod and cone cell death and regeneration in the adult albino zebrafish (Danio rerio) retina." J Neurobiol **44**(3): 289-307.

Vinothkumar, S., S. Rastegar, et al. (2008). "Sequential and cooperative action of Fgfs and Shh in the zebrafish retina." Developmental biology **314**(1): 200-214.

Vlachos, P. and B. Joseph (2009). "The Cdk inhibitor p57(Kip2) controls LIM-kinase 1 activity and regulates actin cytoskeleton dynamics." Oncogene **28**(47): 4175-4188.

Wall, D. S., A. J. Mears, et al. (2009). "Progenitor cell proliferation in the retina is dependent on Notch-independent Sonic hedgehog/Hes1 activity." The Journal of cell biology **184**(1): 101-112.

Wallace, V. A. (1999). "Purkinje-cell-derived Sonic hedgehog regulates granule neuron precursor cell proliferation in the developing mouse cerebellum." Current biology : CB **9**(8): 445-448.

Wallace, V. A. (2008). "Proliferative and cell fate effects of Hedgehog signaling in the vertebrate retina." Brain research **1192**: 61-75.

Wallace, V. A. (2008). "Proliferative and cell fate effects of Hedgehog signaling in the vertebrate retina." Brain Res **1192**: 61-75.

Wan, J., H. Zheng, et al. (2007). "Sonic hedgehog promotes stem-cell potential of Muller glia in the mammalian retina." Biochemical and biophysical research communications **363**(2): 347-354.

Wang, Y., G. D. Dakubo, et al. (2005). "Retinal ganglion cell-derived sonic hedgehog locally controls proliferation and the timing of RGC development in the embryonic mouse retina." Development **132**(22): 5103-5113.

Wang, Y. P., G. Dakubo, et al. (2002). "Development of normal retinal organization depends on Sonic hedgehog signaling from ganglion cells." Nat Neurosci **5**(9): 831-832.

Wang, Y. P., G. Dakubo, et al. (2002). "Development of normal retinal organization depends on Sonic hedgehog signaling from ganglion cells." Nature neuroscience **5**(9): 831-832.

Wechsler-Reya, R. J. and M. P. Scott (1999). "Control of neuronal precursor proliferation in the cerebellum by Sonic Hedgehog." Neuron **22**(1): 103-114.

Wehman, A. M., W. Staub, et al. (2005). "Genetic dissection of the zebrafish retinal stem-cell compartment." Developmental biology **281**(1): 53-65.

Westerfield, M. (2000). The zebrafish book. A guide for the laboratory use of zebrafish (Danio rerio).

Wetts, R., G. N. Serbedzija, et al. (1989). "Cell lineage analysis reveals multipotent precursors in the ciliary margin of the frog retina." Developmental biology **136**(1): 254-263.

Wetts, R., G. N. Serbedzija, et al. (1989). "Cell lineage analysis reveals multipotent precursors in the ciliary margin of the frog retina." Dev Biol **136**(1): 254-263.

Willbold, E., A. Rothermel, et al. (2000). "Muller glia cells reorganize reaggregating chicken retinal cells into correctly laminated in vitro retinae." Glia **29**(1): 45-57.

Xue, X. Y. and W. A. Harris (2011). "Using myc genes to search for stem cells in the ciliary margin of the Xenopus retina." Developmental neurobiology.

Yamaguchi, M., F. Imai, et al. (2010). "Mutations in N-cadherin and a Stardust homolog, Nagie oko, affect cell-cycle exit in zebrafish retina." Mechanisms of development **127**(5-6): 247-264.

Yamaguchi, M., N. Tonou-Fujimori, et al. (2005). "Histone deacetylase 1 regulates retinal neurogenesis in zebrafish by suppressing Wnt and Notch signaling pathways." Development **132**(13): 3027-3043.

Yokoo, T., H. Toyoshima, et al. (2003). "p57Kip2 regulates actin dynamics by binding and translocating LIM-kinase 1 to the nucleus." The Journal of biological chemistry **278**(52): 52919-52923.

Zhang, L., H. M. El-Hodiri, et al. (2003). "Targeted expression of the dominant-negative FGFR4a in the eye using Xrx1A regulatory sequences interferes with normal retinal development." Development **130**(17): 4177-4186.

Zhang, X. M. and X. J. Yang (2001). "Regulation of retinal ganglion cell production by Sonic hedgehog." Development **128**(6): 943-957.

Zuber, M. E., G. Gestri, et al. (2003). "Specification of the vertebrate eye by a network of eye field transcription factors." Development **130**(21): 5155-5167.